The role of tapasin in MHC class 1 antigen presentation

Thesis

THE ROLE OF TAPASIN IN MHC CLASS I ANTIGEN PRESENTATION

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ABSTRACT

MHC class I molecules are heterotrimeric complexes of a polymorphic MHC class I heavy chain, beta-2 microglobulin (β_2 M) and a short peptide. These complexes present self and foreign peptides to host effector cells. This allows for monitoring of the internal milieu of cells through representation of the protein breakdown products (peptides) within MHC class I complexes at the cell surface.

This thesis examines the role of the endoplasmic reticulum resident MHC class I chaperone tapasin in this process. The initial characterisation was undertaken at a genetic level, identifying polymorphic variants of human tapasin. A search for linkage with MHC class I and II genes was carried out, with linkage only identified for the nearest MHC class II loci. The limited genomic variation was further analysed in primate tapasin and 15 amino acid differences from human tapasin were identified. Further work was directed at developing a functional test for the role of tapasin in MHC class I presentation, so that such differences could be analysed.

A model system was set up that examined the role of tapasin upon previously identified tapasin dependent and independent alleles (B*4402 and B*2705 respectively). A thermostability assay was developed that assessed the peptide cargo of B*4402 and B*2705 within cells that were competent or deficient for tapasin. This work identified that tapasin was important for the optimal assembly of both B*4402 and B*2705. Tapasin was shown to improve both the rate and extent of optimal peptide acquisition. More detailed studies showed that the improvement in the thermostability of such complexes could occur independently of tapasin for B*2705, although to a lesser extent to that seen in the presence of tapasin. B*4402 was unable to load optimal peptides in the absence of tapasin, although it was shown that these B*4402 complexes were unlikely to be peptide empty as their thermostability fell further upon TAP blockade. When the loading of these alleles was assessed in the presence of a soluble tapasin construct that did not abridge the MHC class I complexes to TAP, peptide loading was achieved but the thermostability of such complexes was reduced when compared to full length tapasin. This suggested that the association of nascent MHC class I molecules with the TAP complex is necessary for maximal optimisation of the MHC class I complex peptide cargo. Finally, a single point mutation was identified that permitted tapasin independent loading of B*4402. This single change at position 116 was identical to that seen in the allele B*4405. B*4402 was shown to achieve a greater thermostability than B*4405 in the presence of tapasin. B*4405 was able to load optimal peptides in the absence of tapasin, with a further improvement in the presence of tapasin. Both B*2705 and B*4405 were less thermostable than B*4402 in the presence of tapasin.

These experimental observations have led to a reassessment of the multiple functions of tapasin in facilitating peptide loading and a consideration of the molecular mechanisms that may permit such allele specific interactions.

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AUTHORSHIP DECLARATION

I declare that the work undertaken in this thesis is my own and has not been submitted for any other degree. Experimental work undertaken by collaborators and presented in this thesis is detailed below.

Methods
C.A. Peh constructed all the B*4402 and B*2705 cell lines used in this thesis.
A. Purcell constructed the B*4405 cell lines used in this thesis

Results
-Section 3
S Bevan undertook the CSGE analysis of tapasin and sequenced the initial samples when a bandshift was identified.
M.Bunce characterised the MHC class I alleles of the control samples.
-Section 4
C.A.Peh undertook the radioiodination experiment seen in Figure 4.4.1

Aspects of this work have been led to the following peer reviewed publications:

Williams A. P., Bevan S., Bunce M, Houlston R., Welsh K. I., Elliott T.
Identification of novel Tapasin polymorphisms and linkage disequilibrium to MHC class I alleles. Immunogenetics. 2000 Nov;52(1-2):9-11.

Williams A., Peh C.A., Elliott, T.
The cell biology of MHC class I antigen presentation. Tissue Antigens. 2002 Jan;59(1):3-17. Review

Williams A. P., Peh C.A., Purcell A.W., McCluskey J., Elliott, T.
Optimization of the MHC class I peptide cargo is dependent on tapasin.
Immunity. 2002 Apr;16(4):509-20.

Gao B, Williams A., Sewell A.,Elliott T.
Generation of a functional, soluble tapasin protein from an alternatively spliced mRNA.
Genes and Immunity. 2004 Mar;5(2):101-108

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ABBREVIATIONS

ATP	Adenosine Triphosphate
β_2M	β_2-microglobulin
BCR	B cell receptor
BFA	Brefeldin A
bp	Base pair
CAST	Castanospermine
CD	Cluster of differentiation
CpG	Unmethylated cytosine-guanine
CLIP	Class II-associated invariant chain peptide
CTL	Cytotoxic T lymphocyte
CSGE	Conformation specific gel electrophoresis
Cys	Cysteine
dMJ	Deoxymannojirimycin
DNA	Doxyribonucleic acid
DRIP	Defective Ribosomal products
dNTP	Deoxynucleoside Triphosphate
EDEM	ER degradation enhancing α-mannosidase-like protein
EDTA	Ethylene Diamine Tetra-acetic acid
EndoH	Endoglycosidase H
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
ERAP	Endoplasmic reticulum associated aminopeptidase
ERGIC	Endoplasmic reticulum-golgi intermediate compartment
ERM I	Endoplasmic reticulum mannosidase I
ERM II	Endoplasmic reticulum mannosidase II
FACS	Fluorescent activated cell scanning
FCS	Foetal calf serum
FRET	Fluorescent energy transfer
Glc	Glucose
GlcNAc	N-acetyl glucosamine
GS I	Glucosidase I
GS II	Glucosidase II
HC	Heavy chain
HIV	Human Immunodeficiency virus
HLA	Human Leucocyte antigen
IFNγ	Interferon Gamma

Ii	Invariant chain
Kb	Kilobase of DNA
kDa	Kilodalton
Man	Mannose
Mb	Megabase of DNA
Met	Methionine
MFI	Mean Fluorescent Intensity
MHC	Major Histocompatibility complex
NBD	Nucleotide binding domain
NK	Natural Killer cell
NP40	NonidetP-40
PBS	Phosphate buffered Saline
PCR	Polymerase Chain reaction
PE	Phycoerythrin
PR	Peptide receptive complex
PSA	Puromycin sensitive aminopeptidase
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute cell culture medium
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel electrophoresis
SSP	Sequence specific priming
TAP	Transporter associated with Antigen Processing
Tpn	Tapasin
TPPII	Tripeptidyl peptidase II
UGGT	UDP-glucose:glycoprotein glucosyltransferase

CHAPTER 1 INTRODUCTION

1.1 AN OVERVIEW OF THE IMMUNE SYSTEM.

The immune system of man is an evolutionary masterpiece of engineering, designed to protect the host from a panoply of invading micro-organisms. It exists to provide an immediate response to an enormous variety and number of pathogenic viruses, bacteria, fungi and parasites. Critically, it is able to build upon this initial encounter to protect the host (and its offspring) from similar or recurrent life-threatening infections. Such immune responses require the host to recognise the foreign molecules of pathogens and thereby effect a counter response to eliminate the pathogen with negligible harm to the host. The foreign molecules that generate the *anti*-patho*gen* response are termed *antigens*. The immune recognition of such antigens is carried out by lymphocytes and their accessory cells. The requirement to both identify all potential foreign molecules and distinguish these from self molecules has been achieved in a variety of ways. Certain 'signature' molecules are expressed by foreign micro-organisms and these provide the immune system with a predictable, fixed and discriminating signal in which to identify potential invasion (Underhill, 2003). The Toll receptors represent the best appreciated recognition receptors for this category of antigen and these are principally expressed on the surface of accessory antigen presenting cells. These antigens include bacterial DNA (CpG), viral RNA

(dsRNA) and parasitic proteins (flagellin). This 'hard wired' recognition system (often termed Pattern Recognition) is considered part of the innate immune system as it has little ability to adapt to change or mutation within the pathogen, is activated early within host defence and has no capacity to improve upon subsequent exposure (Medzhitov and Janeway, 2000).

Immune responses that are directed against unique antigen patterns of a pathogen are usually referred to as adaptive immune responses (often termed Specific Recognition). This type of response has two key attributes, namely specificity and memory. This latter property allows for an enhanced response to that antigen upon a secondary exposure. This type of adaptive immune response is mediated by lymphocytes. These cells have evolved in concert with the innate systems of phagocytic and plasma enzyme based host defence, to provide an integrated immune recognition and response unit. Indeed, there exists mutual dependence upon these sub-systems in affording the optimum response to micro-organisms (Fearon and Locksley, 1996).

Lymphocytes are derived from a lymphoid stem cell within the mammalian bone marrow. This precursor cell gives rise to two principal lymphocyte subsets, namely the B and T cells. B cells are generated in the bone marrow and display cell surface immunoglobulin molecules that are capable of recognising foreign proteins. This immunoglobulin can be secreted to provide a soluble immune recognition defence and this can be an important component of the adaptive

immune response to certain invading micro-organisms. The T cells are generated in the thymus gland following export from the bone marrow. They can subsequently be identified in peripheral blood, lymphoid organs and tissue sites where they are divided into 2 subsets based upon the expression of certain cell surface co-receptors. The co-receptors are the CD4 or CD8 molecules which assist in the identification of their respective immune recognition molecules. The adaptive responses mediated by both T and B cells are achieved through the rearrangement of germ line genes that produce an almost infinite repertoire of anticipatory B and T cell receptors. The B cell immunoglobulin receptor consist of a heavy and light chain that contribute towards antigen recognition. Each chain has a constant region and a variable region which defines its uniqueness and specificity. The T cell receptor is similar in concept, having 2 paired chains each with a constant and variable region. Both receptors have a similar configuration of germ line genes that encode for their respective configuration of B and T cell receptors (BCR and TCR). Their receptor loci have a variable and constant block of genes. The variable block is made from variable (V), joining (J) and diversity (D) gene segments. For the BCR, 250-1000 V, 10 D and 4 J segments exist for the heavy chain with over 250 segments for the 2 possible light chains. The T cell receptor exists in 2 forms, either utilising α and β gene loci (majority of peripheral T cells) or γ and δ gene segments. The α locus contains approximately 100 variable and 50 joining regions, whilst the β region has 50 V, 2 D and 12 J regions. These germline segments assemble to form a VDJ/VJ variable region. This combinatorial association and joining greatly

diversifies the potential repertoire. This is further broadened by the processes of N-region diversity and junction flexibility in both BCR and TCR's and a unique process of somatic hypermutation for the BCR. The end result of this genetic flexibility is a receptor repertoire estimated at 10^{15}-10^{19}. This anticipatory capacity exceeds the total number of lymphocytes with a host but permits an extremely broad repertoire of receptors upon B and T cells for subsequent recognition of invading micro-organisms (Paul, 1993).

The antigen presentation molecules that are recognised by the receptors of the adaptive immune system are specific for each form of antigen and often its cellular location. B cells recognise intact protein through their cell surface B cell receptors or secreted immunoglobulin. The recognition is determined by the shape and fit congruity between the antigen and the BCR heavy and light chains. T cells recognise antigens in a fundamentally different manner. CD8 positive T cells recognise small protein fragments (peptides) presented by cell surface molecules termed Major Histocompatibility Class I (MHC I) complexes (Townsend and Bodmer, 1989). Typically these are the classical and non classical MHC class I complexes termed HLA-A, B and C and HLA -E, F, G respectively (see section 1.2). CD4 T cells recognise protein fragments presented by cell surface molecules termed Major Histocompatibility Class II complexes (Cresswell, 1994). Both antigen presentation molecules are encoded on a region of chromosome 6 that is dense for genes involved in immunological processes. MHC class I molecules principally present peptides from the

intracellular environment of the antigen presenting cell whilst MHC class II complexes present peptides derived from the extracellular environment. These MHC molecules are polymorphic at a population level and polygenic at an individual level (Parham and Ohta, 1996). Such diversity is believed to permit presentation of a multitude of antigens from the infinite number of micro-organisms a species may encounter. This immune response at a population level is an important concept in understanding the influences that have shaped the immune system. Finally, it has become recently appreciated that a third family of antigen presentation receptors are important in host protection by T cells. This family of CD1 molecules presents lipid antigens to the adaptive immune system, increasing further the breadth of micro-organism targeting (Dutronc and Porcelli, 2002) (Figure 1.1).

Once a pathogen has been recognised by the immune system, two processes occur. The first is a period of proliferation (originally termed clonal selection) where the T and B cells having receptors specific for the invading organism receive a positive signal to expand their number from its low precursor frequency. For B cells this is accompanied by a genetic program that permits improvements to be made in the affinity of the BCR for the stimulating antigen. For T cells this is not possible, but an improved avidity for the antigen is thought to occur through the redistribution and aggregation of the TCR within certain domains of the plasma membrane (Farmery et al., 2000; Slifka and Whitton, 2001). The second stage is that of an effector response directed at clearing the inciting pathogen.

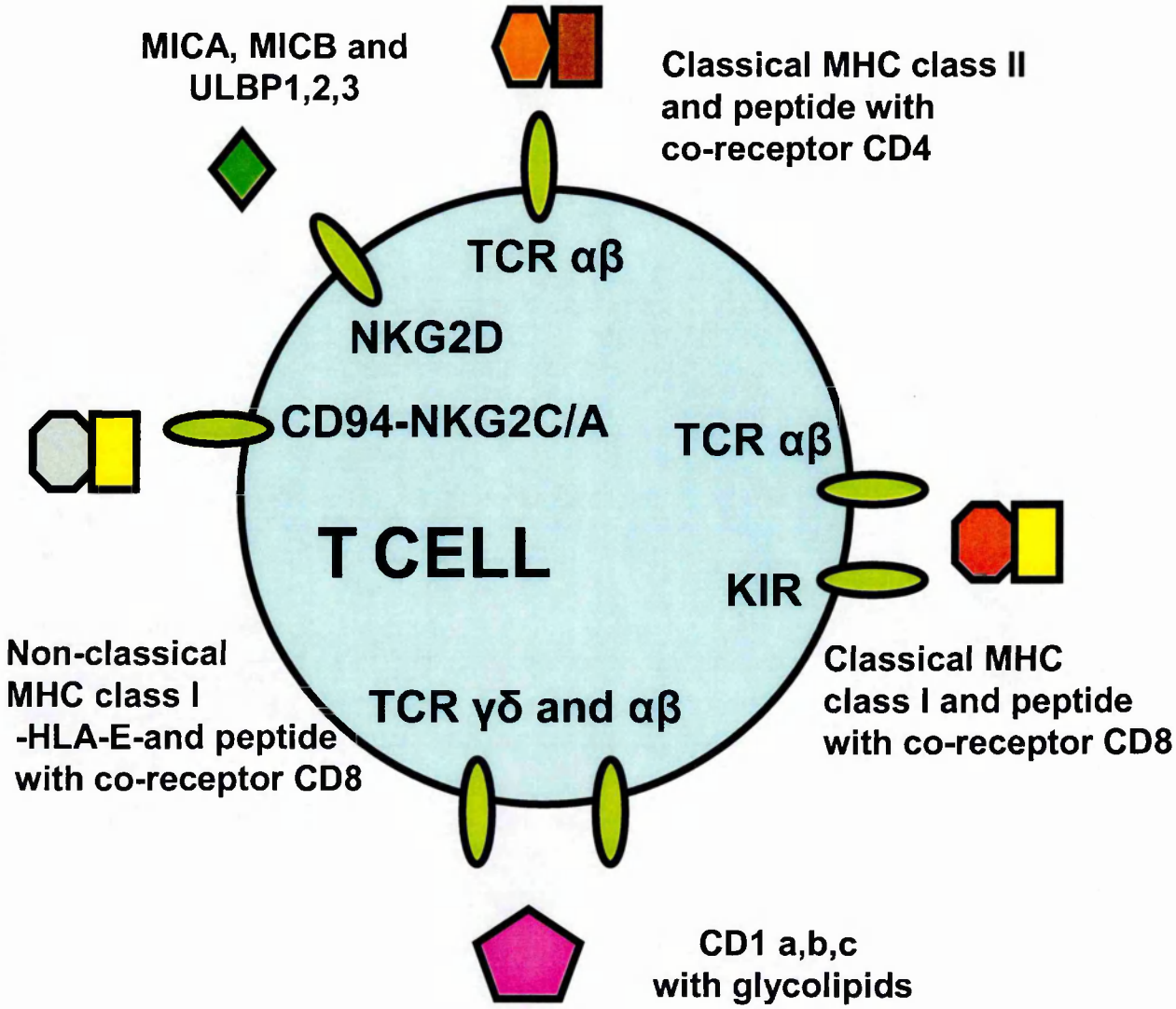


Figure 1.1 - Antigen Presentation

Multiple presentation strategies are available to present antigens to T cells through polymorphic and non polymorphic receptors.

For B cells this may be capture and removal of a harmful toxin following secretion of the BCR, fixation to a micro-organism and engagement of plasma enzyme systems (complement) or uptake of the organism through membrane bound BCR for presentation to T cells. The T cell response may involve the secretion of molecules to aid B cell development, attract accessory cells or promote cell killing. In the case of CD8 T cells, direct cytotoxicity of the infected cell may be undertaken through the release of lytic granules and enzymes (Catalfamo and Henkart, 2003). Alternatively cell surface Fas ligand may mediate cellular cytotoxicity through ligation of the target cell's Fas receptor (CD95) (Berke, 1995). Following control and/or clearance of the pathogen, a state of adaptive memory is maintained. For B cells this is demonstrated by the continued production of high affinity antibody that is specific for the prior organism. T cells specific for a previously seen pathogen maintain themselves at a higher precursor frequency and acquire the capacity to exert an effector response at an earlier time point upon subsequent exposure.

The importance of each component of this integrated system is apparent when natural deficiencies of certain components are described in man. The selective absence of B cells, CD4, CD8 T cells, complement, phagocytes or MHC class I/II molecules all have characteristic phenotypes that highlight their particular niche in eliminating certain pathogens (Fischer, 2002). The role of tapasin in the cell biology of MHC class I presentation to CD8 T cells is considered with this broad canvas in mind.

1.2 THE MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) GENES

The major Histocompatibility complex genes encode for molecules that are fundamental to self/non self discrimination. Their initial description and terminology relates to their identification through allogenic transplantation studies in mice. This work initially involved the study of tumour transplant rejection by Snell and Gorer (Gorer et al., 1948) and later skin transplantation by Medawar (Billingham et al., 1953). It was appreciated that graft refection was immunologically mediated and a series of experimental rabbit, guinea pig and mouse systems were set up to identify the mechanisms involved (Levine et al., 1963; McDevitt and Sela, 1965). Immunogenetic studies using highly inbred strains of mice defined a region called the major Histocompatibility complex on chromosome 17 in mice and chromosome 6 in humans (Klein, 2001). This genetic region dictated the compatibility between different strains of mice for transplant acceptance or rejection. At the same time McDevitt and others were investigating immune response (IR) genes that determined whether a strain would respond to polypeptide antigens. These IR genes were localised to the same MHC region (McDevitt and Chinitz, 1969). It is now appreciated that both immune responses to foreign polypeptides (i.e. viral, bacterial), non-self transplants and self antigens are directed through the MHC class I and II proteins of the MHC.

The human MHC is located on the short arm of chromosome 6 and is divided into 3 areas. The MHC class I region contains the MHC class I genes, MHC class II area the MHC Class II genes and the MHC class III area other immunologically relevant (and non relevant) genes (Kumanovics et al., 2003). The human MHC region extends over 4 Mb with the MHC class I region centromeric, the extended MHC class II region telomeric and the MHC class III region between the two. The MHC class III region is about 700kb in length and contains immune genes relating to the complement system and tumour necrosis family (Beck and Trowsdale, 2000) (Figure 1.2). The MHC class I and II region will be discussed separately.

1.2.1 MHC CLASS I REGION

The MHC class I region extends from the HLA-F locus at the telomeric end of the short arm of chromosome 6 to the HLA–B locus, a distance of approximately 1.4MB. There are six functional genes: HLA-A, B, C, E, G, and F within this region (Kumanovics et al., 2003). The HLA A, B and C genes are highly polymorphic and termed classical MHC as they were responsible for the original histoincompatibility observations (termed major Histocompatibility antigens) (Klein, 1986; Klein, 2001). Recently a new taxonomy of mammalian MHC class I molecules has been proposed (Hughes et al., 1999). Previously the classical MHC class I molecules have been termed class 1a whilst the non-classical have

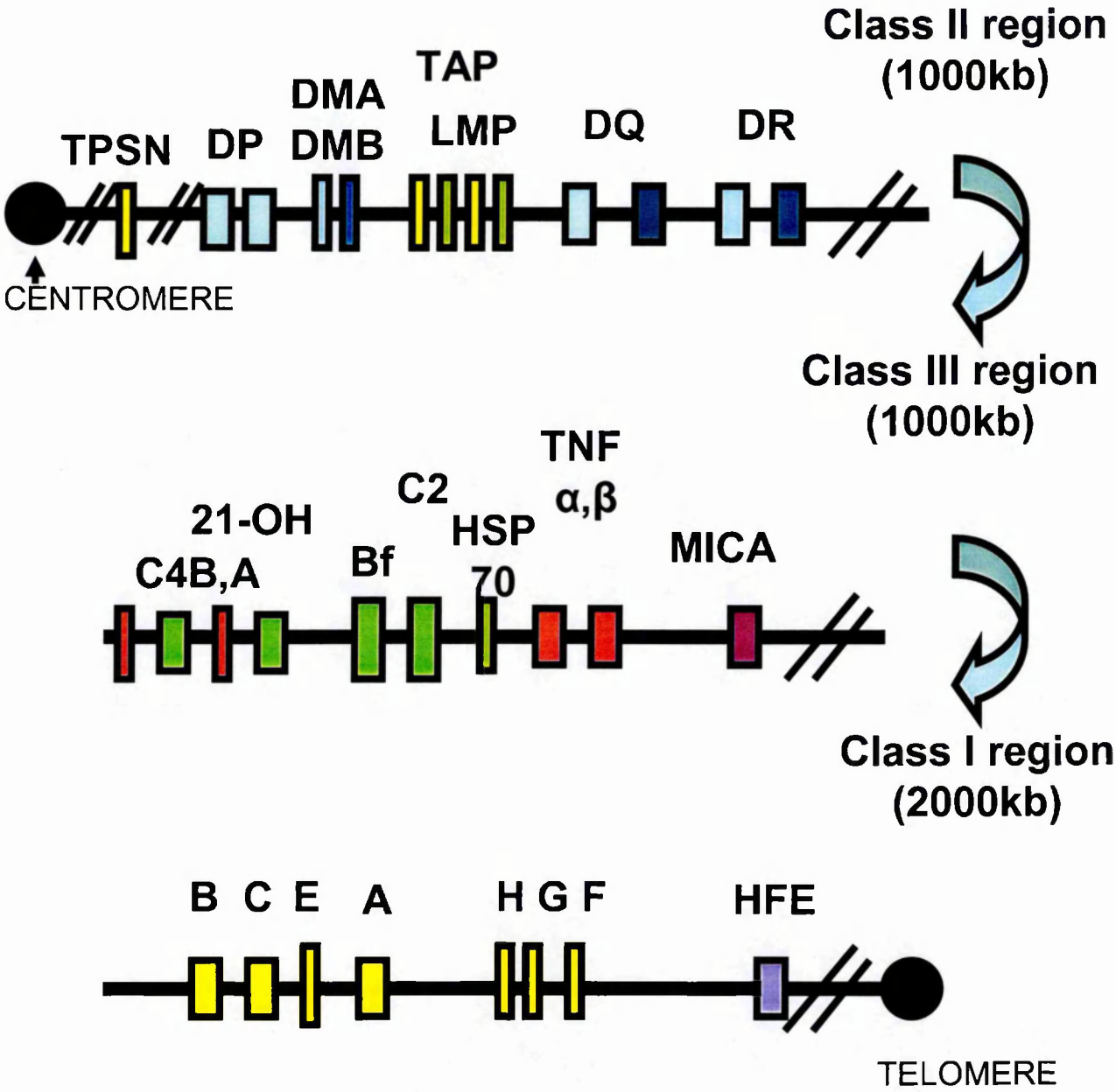


Figure 1.2 – Schematic representation of the human MHC complex on the short arm of chromosome 6

The genes involved in MHC class I antigen presentation are coloured in yellow whilst those of the class II pathway are in blue. Within the MHC class III region other genes of immunological importance are found (complement in green)

been termed class 1b. Due to the discovery of gene families within the MHC possessing a greater divergence from class Ia than previous class Ib genes (i.e. MIC), and the recognition of other class I related genes outside of the MHC region, a new nomenclature has been proposed for human and mouse MHC class I molecules (Table 1.2.1). This extends the previous class 1a and class 1b categories with a class 1c group including MICA, MICB and the haemachromatosis gene (HFE) which are on chromosome 6 and a class 1d which are not (i.e. CD1and the neonatal Fc receptor [FCRn]).

The MHC class 1a genes encode for the MHC class I heavy chain which non-covalently associates with β_2–microglobulin (β_2M) to form the MHC class I complex. The genes encoding the HLA-A, B and C heavy chain have very a similar exon-intron organisation in which separate exons encode the signal peptide, the three extracellular domains (α_1, α_2 and α_3), the transmembrane region and cytoplasmic tail. Exons 2 and 3 encode for the α_1 and α_2 regions of the mature protein, providing the peptide binding site. Exon 4 encodes the α_3 region of the mature protein which interacts with β_2M. This association is recognised by the CD8 co-receptor of MHC class I restricted T cells. The polymorphism between the various HLA-A, B and C alleles is primarily located in exons 2 and 3 and contributes to the specificity of the peptide binding groove (Marsh et al., 2000). The α_1 and α_2 domains consist of two α–helices that rest upon a sheet of eight β–strands, thereby forming the peptide binding groove. The main purpose of the MHC Class I complex is to present peptides of 8-9

	CLASS 1A	CLASS 1B	CLASS 1C	CLASS 1D
Human	HLA-A	HLA-E	MICA	FcRn
	HLA-B	HLA-F	MICB	CD1
	HLA-C	HLA-G	HFE	
Mouse	H-2K	H-2Q	HFE	FcRn
	H-2D	H-2T		CD1
	H-2L	H-2M		

TABLE 1.2.1 – A new taxonomy of MHC class I molecules

The abbreviations used are FcRn for the Fc receptor gene for HFE for the haemachromatosis gene.

amino acids in length within this groove to CD8 positive T cells (Bjorkman et al., 1987a; Bjorkman et al., 1987b; Townsend et al., 1985; Zinkernagel and Doherty, 1974). In addition to this function such MHC class I peptide complexes are targets for NK cells which possess receptors that identify polymorphic resides in the α_1 region (Sawicki et al., 2001). This is particularly evident for HLA C complexes at amino acid (aa) residues 77-80 and HLA A and B at positions 77-83 (Boyington et al., 2001). The non-classical MHC class I loci encode for less polymorphic heavy chains that all have a similar structure and requirement for β_2M association. These proteins have a limited tissue distribution in comparison to classical MHC which is expressed on virtually all nucleated cells. HLA-E presents peptides derived from the signal sequence of other MHC class I proteins to natural killer cells (NK cells) expressing CD94/NKG2 receptors (Braud et al., 1998a). More recently HLA-E has also been shown to present foreign antigen to $\alpha\beta$ T cells (Heinzel et al., 2002). HLA-G is able to bind a wider variety of peptides but has a limited tissue expression confined to the extravillous cytotrophoblast of the placenta (Le Bouteiller and Blaschitz, 1999). Very little is known about HLA-F. Its expression is mainly restricted to B cells, being retained in the ER as a TAP bound complex (Wainwright et al., 2000). More recently HLA-F has been identified on the cell surface of extravillous trophoblasts that have invaded the maternal deciduas (Ishitani et al., 2003). In addition to the classical and non-classical genes, the MHC class I region also contain two MIC genes (MHC class I chain related, MICA and MICB). These are polymorphic; do not require an association with β_2M and their cell surface expression is restricted

to intestinal epithelium under normal conditions (Stephens, 2001). They are recognised by an activating receptor complex (NKG2D/DAP10) that is present on NK cells and some T cells.

Non-class I genes outnumber the MHC class I genes within the MHC class I region. In the 600Kb segment between HLA-C and HLA-E there are at least 20 functional non-MHC class I genes (Consortium, 1999). These include transcription factors, a tyrosine kinase, helicase and a group of genes potentially involved in the pathogenesis of psoriasis (Oka et al., 1999). Unlike the class I genes, the non-class I genes in this region are orthologous among different species from different mammalian orders (Figure 1.2.1a). The class I genes occupy the same intergenic regions but are not necessarily related across species. The framework hypothesis suggests that the non-class I genes represent a framework that was filled by the expanding MHC class I genes. Both human and mouse class I regions have a class I family that is missing in the other MHC. There are no MIC genes in the mouse and no H2/10 like genes humans. It is interesting to note that there are no other MHC Class I specific genes in the MHC class I region; with tapasin, TAP and the proteasome subunits encoded elsewhere within the MHC. This is in contrast to the chicken MHC where the MHC class I region includes both TAP genes between the 2 class I genes, extending over a small region of less than 15Kb (Kaufman et al., 1999) (Figure 1.2.1b). These intergenic distances are important in determining the probability of co-inheritance of certain gene families. The apparent linkage

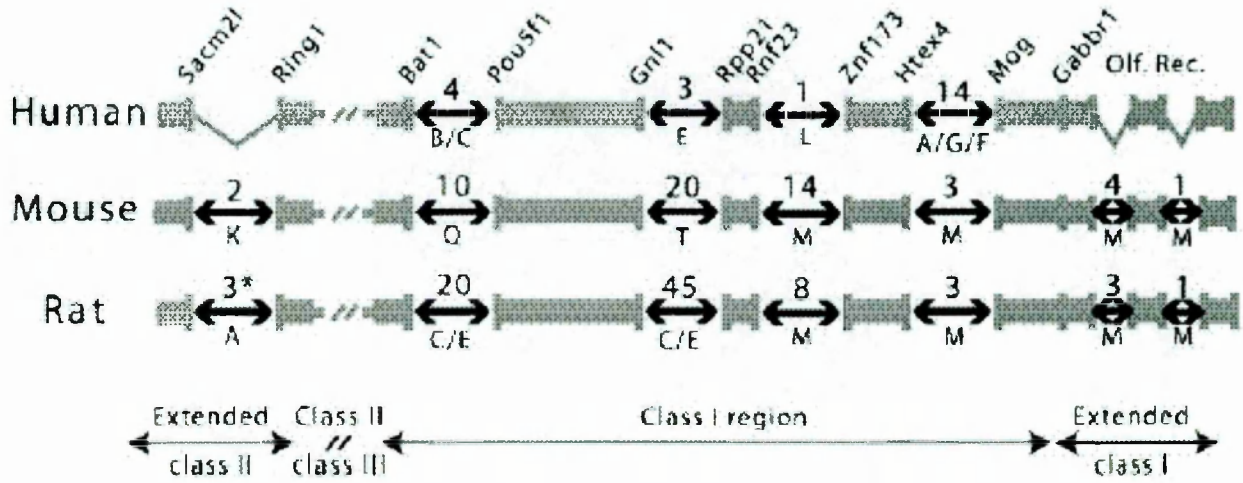


Figure 1.2.1a – Comparison of human, mouse and rat class I regions

The gray boxes represent the conserved framework regions that encode the non-MHC class I genes. The figures represent the class I gene content of the 129/SvJ mouse and the published human sequence.
This figure is taken from Kumanovics et al, Ann. Rev. Immunol. 2003.21:629-57

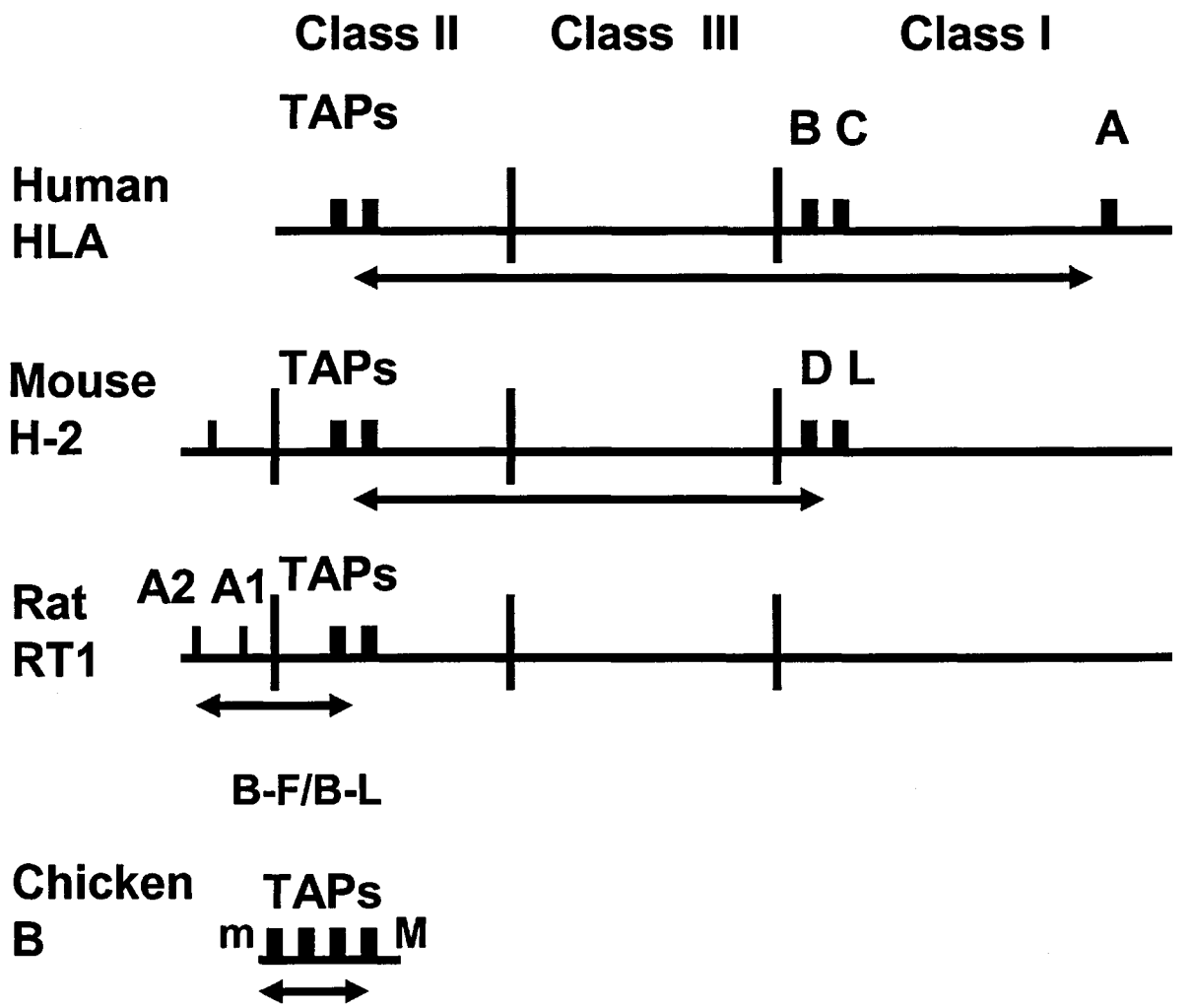


Figure 1.2.1b - Organization of human, mouse rat and chicken MHC regions.

The human HLA complex extends over almost 4 Mb of DNA between the TAP genes and classical Class I genes. The H-2 complex and RT1 regions extend over 2Mb and 300Kb distances respectively. The chicken B locus has the classical Class I genes flanking the TAP genes and separated by a distance of only 30 nucleotides.

between 2 genes is occasionally in excess of what would be expected by chance and such linkage disequilibria are thought to represent a beneficial interaction between such genes. This area of linkage has proven to be important within the MHC class I, II and II regions where particular haplotypes have been recognised (Price et al., 1999). The relevance of such co inheritance has been detailed in the rat MHC where a particular MHC class I allele is co-inherited with a peptide transporter that is able to correctly furnish the co-inherited class I complex with appropriate peptides (Powis et al., 1992). Such clear examples have not emerged in the study of human MHC class I complexes.

1.2.2 MHC CLASS II REGION

The MHC class II region extends from HLA-DR through to DP (Figure 1.2.2a). The MHC class II molecules that present peptides to CD4 positive T cells are encoded by 3 loci within this region. These are HLA-DP, DQ and DR, each encoding separate α and β genes. The DR genes encode one DR α gene and up to 4 DR β genes. DP and DQ encode a single functional α and β gene. The α chains of each loci associate with the β chains of their own family. A high degree of polymorphism is seen in class II molecules. This is mostly in the genes of each β loci. These polymorphisms are not random but occur in regions of the molecule around the peptide binding groove.

The products of the MHC class II genes are heterodimers of heavy (α) and light (β) glycoproteins. Each chain has 5 domains: 2 extracellular globular domains, a connecting peptide, a transmembrane domain, and a cytoplasmic domain. The α_2 and β_2 extracellular domains have the structural characteristics of an immunoglobulin domain. The α_1, β_1 and β_2 domains are disulphide linked whilst the α_1 domain is not. X ray crystallography has shown that the class II molecule has a peptide backbone composed of 8 strands of anti-parallel β sheets, forming a floor, and 2 anti-parallel helical regions as the sides (Stern and Wiley, 1994). The structure of the MHC class II peptide binding groove is remarkably similar to that of the MHC class I structure despite being conformed by 2 separate chains. One key difference between the class I and class II structures is the relatively open end of the groove which allows the class II molecule to except larger peptides than that of MHC class I molecules (Fremont et al., 1998; Fremont et al., 1996).

Other MHC class II molecules encoded in the MHC class II region include HLA-DM (DM) and DO. These are relatively non polymorphic molecules that do not bind peptide. These contain sorting signals in their cytoplasmic, tails directing the molecules to the endosomal compartments where they promote antigenic peptide loading (Alfonso and Karlsson, 2000).

The MHC class II region also contains genes involved in MHC class I antigen presentation. These are the TAP 1, TAP 2, LMP2 and LMP7 genes. The first 2

genes encode for a heterodimeric ATP dependent peptide transporter that transfers peptides from the cytoplasm into the ER for MHC class I assembly (Uebel and Tampe, 1999). The latter 2 genes are inducible catalytically active subunits of the proteasome (Van den Eynde and Morel, 2001). These form part of the 'immunoproteasome' that generates antigenic peptides from polypeptide precursors in the cytoplasm.

The extended MHC class II region describes a region from KNSL2 to COL11A2 at the centromeric end of the MHC, approximately 500kb from DP (Beck and Trowsdale, 1999; Herberg et al., 1998a). This region contains the tapasin gene and is syntenic with the extended MHC class II region in mice (Figure 1.2.2b).

1.3 ANTIGEN PRESENTATION

1.3.1 MHC CLASS I PRESENTATION

MHC class I A, B and C complexes are expressed on the surface of virtually all nucleated cells. They exist as a heterotrimeric complex of MHC class I heavy chain (HC), β_2M and peptide. This trimeric structure is the ligand recognised by MHC class I restricted CD8 T cells and MHC class I reactive NK cells (Pamer and Cresswell, 1998). In the absence of peptide or β_2M no partial complexes are able to exit the endoplasmic reticulum (ER) (Arce-Gomez et al., 1978; Baas et al., 1992; Cerundolo et al., 1990).

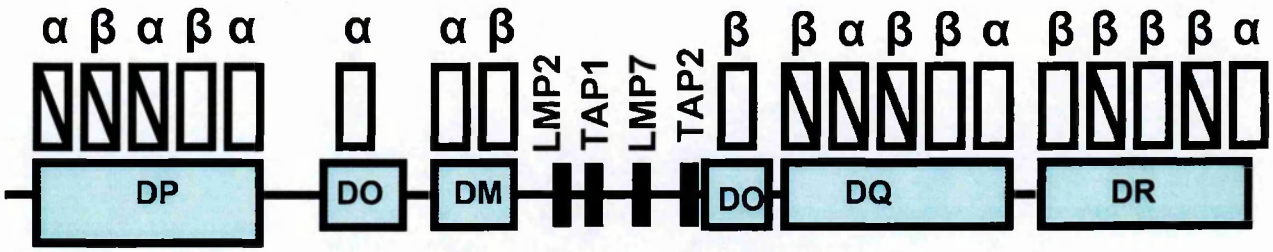


Figure 1.2.2a – Gene content of the human MHC class II region

MHC class II pseudo genes are marked with a crossed box and black boxes represent non-MHC class II genes

Figure 1.2.2b – Gene content of the extended MHC class II region

The region from KNSL2 to COL11A2 at the centromeric end of the MHC is conserved between human and mouse. It contains 15 orthologous genes, including the tapasin gene.

Each HC is initially translated into the ER as a nascent glycoprotein. Within the ER it undergoes assisted folding and disulphide linkage of its cysteine residues within the α_2 and α_3 regions. This folding is assisted by the glycoprotein chaperone calnexin and thioreducatase ERp57 (Bergeron et al., 1994; Farmery et al., 2000; Nossner and Parham, 1995; Sugita and Brenner, 1994). Once correctly folded, the HC may interact with β_2M (Smith et al., 1995). This peptide receptive complex is then able to acquire a peptide within the ER. The binding of peptide stabilises the non-covalent interaction between HC and β_2M permitting egress from the ER (Elliott, 1991; Elliott et al., 1991). Each MHC class I molecule has a peptide binding site which consists of two anti-parallel α-helices overlying a platform of β-strands. The peptide lies in the groove with its N and C termini amino acid side chains interacting with the groove to form a stable, high affinity interaction. The extensive polymorphism that characterises MHC class I gene complex is directed at residues that contribute to the peptide binding groove (Elliott et al., 1993). This amino acid changes alter the pockets within the binding groove or potential hydrogen bond networks that may be set up with certain peptides. These limitations set up particular binding motifs for each allele that are unique to the individual peptide binding grooves. The bound peptide has its anchor residues fill various pockets within the peptide binding groove whilst other amino acid side chains point out of the groove to T cells (Madden, 1995). In the MHC class I peptide binding groove 6 pockets have been identified, namely A, B, C, D, E and F. The A and F pockets interact with the N and C

termini of the bound peptide setting up important hydrogen bonds and salt bridges with the residues in these pockets. The other pockets have similar interaction with the peptide side chains. For human MHC class I alleles a hydrophobic or basic residue is preferred at the C-terminus (Engelhard et al., 2002). The T cell recognises the exposed residues of the peptide and elements of the α-helix (Gao et al., 1997; Garboczi et al., 1996). The peptides are predominantly generated in the cytosol though the action of a protease known as the proteasome (Rock and Goldberg, 1999; Rock et al., 1994). These peptides are then transported into the ER through a heterodimeric, transmembrane ABC transporter known as TAP (transporter associated with antigen processing) (Elliott, 1997; Townsend et al., 1989). This is a constitutive process that can be further increased through the action of interferon gamma (IFN-γ) (Elliott, 1997). The peptide is then loaded onto the awaiting peptide receptive MHC class I molecule. This peptide loading is undertaken through the co-operative actions of at least three MHC class I assembly chaperones that co-associate on TAP (Bouvier, 2003; Momburg and Tan, 2002). These proteins are tapasin, calreticulin and ERp57. When associated with TAP they form an assembly complex which is termed the peptide loading complex (PLC) (Cresswell et al., 1999; Ortmann et al., 1997). Exceptions to this pathway do exist with some MHC class I alleles able to load transported peptides without an interaction with this complex and others able to load peptides in the absence of TAP (Neisig et al., 1996; Wei and Cresswell, 1992). The transported peptides are typically 9-15 amino acids in length with an optimal requirement for MHC class I association of

8-9 amino acids (Shastri et al., 2002). The C terminus of the transported peptides is determined through the action of the proteasome whilst the N terminus may undergo further trimming within the ER (Craiu et al., 1997; Mo et al., 2000). One such ER aminopeptidase has recently been identified (ERAAP) and has been shown to be important in generating the optimal peptide for certain alleles (Saric et al., 2002; York et al., 2002). Once the MHC class I complex is stably loaded with peptide it is able to exit the ER. Its arrival at the cell surface permits T cells to sample the protein content of the cell, identifying 'foreign contamination' amongst the self repertoire of MHC class I peptides. This sampling of endogenous protein is in contrast to the MHC Class II system where sampling of exogenous proteins that access a different peptide loading environment is undertaken (Figure 1.3.1).

In addition to this classical pathway of MHC class I presentation other alternate pathways appear to exist (Reimann and Schirmbeck, 1999). These pathways have similarities to the MHC class II system as they can sample exogenous antigens and present such peptides to CD8 positive T cells. These processes have been termed cross-presentation when referenced to the antigen presenting dendritic cell (Yewdell et al., 1999). There are at least 4 ways in which this non classical presentation may allow MHC class I complexes to acquire peptide. The first appears to involve a phagosome to cytosol pathway which then intersects with the conventional presentation route (Rodriguez et al., 1999). This pathway may utilise a specific transporter from the phagosome to cytosol or follow from a

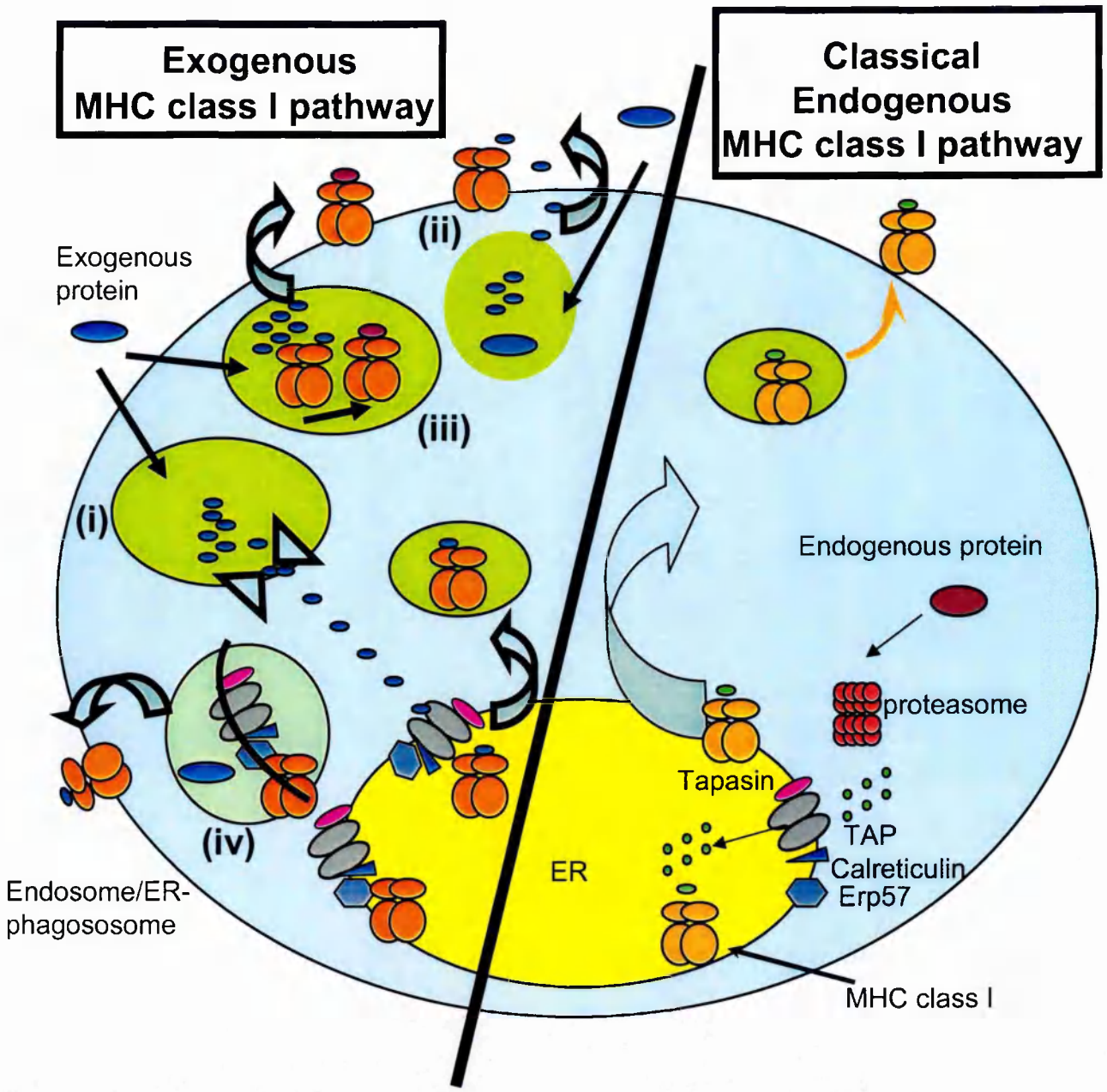


Figure 1.3.1- Endogenous and exogenous MHC class I antigen presentation pathways.

The classical pathway processes endogenous antigen and directs peptide loaded complexes form the ER to the cell surface. The exogenous pathway operates in professional antigen presenting cells through a variety of possible mechanisms. (i) Phagosome to cytosol transfer of exogenous antigen (ii) Regurgitation of endosomally processed antigen to peptide receptive cell surface complexes (iii) Recycling and loading of cell surface complexes within endosomal compartments and (iv) direct trafficking of complexes from the ER to endosomal compartments or through fusion of the ER with a phagosome.

loss of phagosomal membrane integrity. The second manner involves exogenous antigens that have been processed within an endolysomal compartment and then regurgitated into the extracellular space to be bound by peptide receptive cell surface complexes (Pfeifer et al., 1993). The third route relates to the cell surface recycling of MHC class I complexes through endosomal compartments. Such recycling through acid compartments may liberate certain acid sensitive peptides and permit exchange for other peptides within these compartments (Gromme et al., 1999; Schirmbeck et al., 1995; Schirmbeck and Reimann, 1996). Lastly, MHC class I complexes may traffic directly to such endosomal compartments, perhaps through the co-association with invariant chain (Cerundolo et al., 1992; Vigna et al., 1996). Most recently it has been shown that the ER may fuse with a phagosome within dendritic cells and macrophages to generate a new ER-phagosome compartment that is competent for MHC class I assembly and presentation (Ackerman et al., 2003; Guermonprez et al., 2003; Houde et al., 2003). It is believed that these alternate pathways of MHC class I presentation are of fundamental importance in initiating responses through dendritic cells (Heath and Carbone, 2001). Dendritic cell mediated CD8 T cell priming against an intracellular bacteria or virus is thought to require such cross presentation, if such organisms do not have tropism for dendritic cells. Furthermore for some pathogens that interfere with classical antigen presentation systems such alternate pathways provide another approach to CTL (cytotoxic T lymphocyte) generation (Hewitt, 2003).

1.3.2 MHC CLASS II PRESENTATION

MHC class II antigen presentation is analogous to MHC class I presentation in that its primary purpose is to present peptide antigens to T cells. The MHC class II genes are encoded 1Kb centromeric of the MHC class I genes and have comparable polymorphic variation. The MHC class II genes are divided into 3 families DP, DQ, and DP, which in contrast to MHC class I products are only expressed on specialised antigen presenting cells (Beck and Trowsdale, 1999). These are namely dendritic cells, B cells and cells of the monocyte/macrophage lineage. The protein structure of MHC class II molecules is similar to MHC class I molecules with a non covalent association of an α and β chain to provide the antigen binding cleft (Figure 1.3.2). The MHC class II:peptide complex is then expressed at the cell surface for recognition by CD4 positive T cells. These CD4 T cells may then mediate a diverse array of effector or helper functions for both CD8 T cells and B cells.

The MHC class II heterodimer is composed of the transmembrane α and β subunits from the DR, DQ or DP loci. The loci are highly polymorphic with polymorphisms largely confined to the antigen binding pocket of these molecules. (The DR α gene is the exception being essentially monomorphic). The peptide binding cleft is an extended groove with an eight stranded β-pleated sheet forming the floor and 2 α-helical regions forming the walls. The antigenic peptides that are loaded into this groove are derived from foreign and self

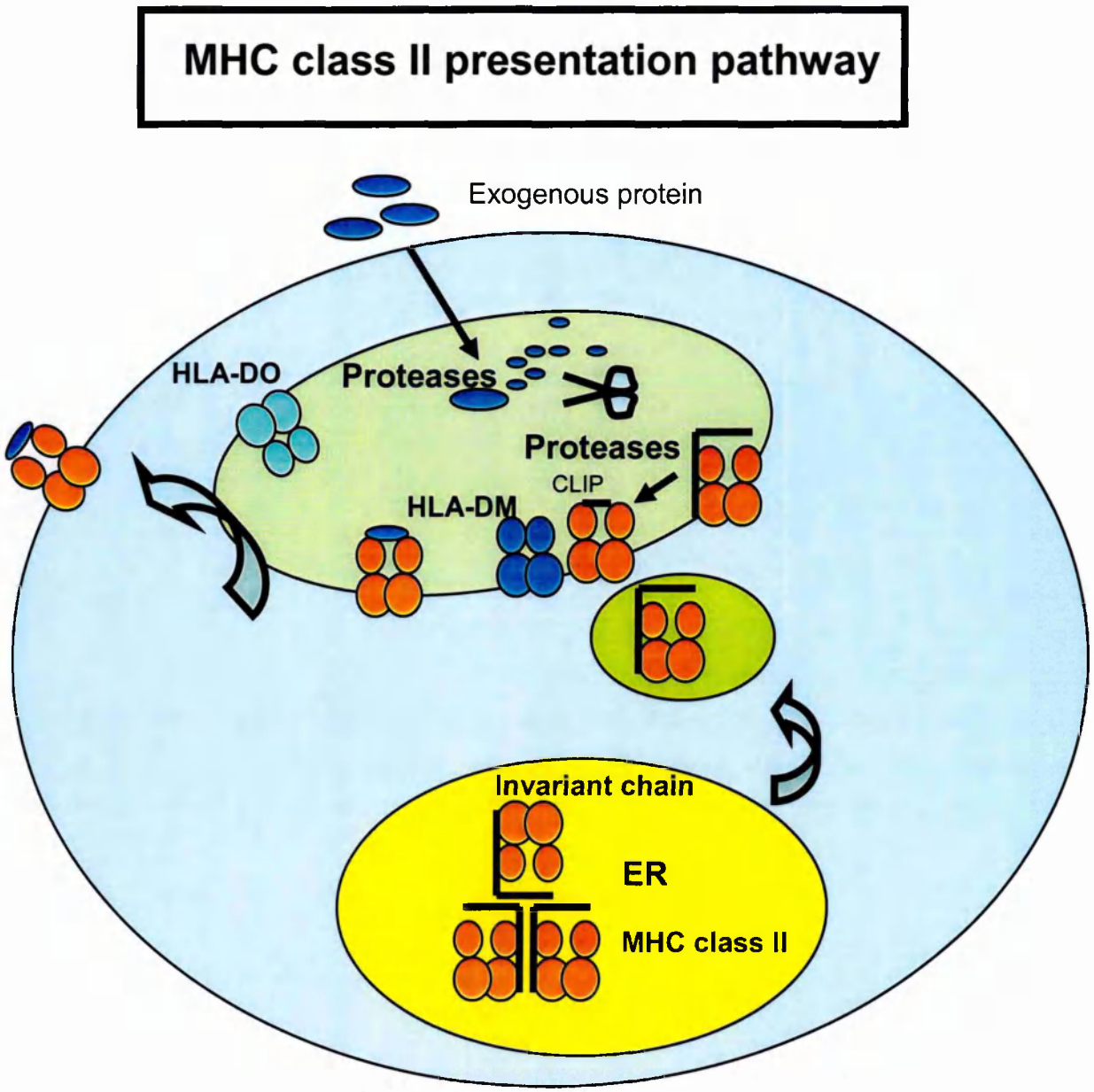


Figure 1.3.2 –MHC class II presentation pathway

A schematic representation of the MHC class II presentation pathway. The nascent MHC class II molecule is loaded in the ER with the Invariant chain (Ii) chaperone. Intersection with the endocytic pathway ensures access to exogenous antigen. Proteases digest Ii and exogenous antigen and HLA-DM subsequently promotes the exchange of CLIP for peptide within the MHC class II loading compartment (MIIC).

proteins. These are typically longer than MHC class I peptides, typically being 10-15 amino acids in length (Rammensee et al., 1995). Their suitability for occupation of the MHC class II groove is dependent upon the hydrogen bond network that is established between the peptide backbone and the floor of the peptide binding groove. Within the peptide binding groove distinct pockets have been identified which accept the peptide side chains of the antigenic peptides. The polymorphisms in the class II structure are located within these pockets and this dictates the allele specificity of peptide binding (Engelhard, 1994).

The MHC class II proteins are initially assembled in the ER. This initially folding and assembly is supported by the glycoprotein chaperones calnexin and BiP (Anderson and Cresswell, 1994; Schaiff et al., 1992). A specific class II accessory molecule termed invariant chain (Ii) also associates with nascent class II within the ER (Roche and Cresswell, 1990). The Ii has a number of important properties and functions. It is a transmembrane glycoprotein with 2 flexible domains. The first domain contains a 21 amino acid sequence (position 80-101) that is known as CLIP (Class II associated Invariant chain peptide). The cytosolic tail contains both an endosomal sorting and ER export signal. These 2 structural features are paramount to the successful loading of MHC class II molecules as the nascent class II molecules all bind to the CLIP region of invariant chain, permitting egress to their respective antigenic peptide loading compartments (Cresswell, 1992). This initial MHC class II $\alpha\beta$Ii complex co-associates as trimers which are subsequently liberated from calnexin and traffic from the ER. The $\alpha\beta$Ii nonamers transverse to the golgi where they are sorted

towards the endocytic route and out of the default secretory pathway to the cell surface. This is accomplished through the recognition of a dileucine motif in the cytoplasmic tail of Ii (Lotteau et al., 1990). The entry of the nascent MH class II complexes in the endosomal system is critical as their peptide repertoire is sampled from internalised proteins that have been modified within the endosomal compartments (Bakke and Dobberstein, 1990; Pieters et al., 1993). This intersection of two trafficking pathways is characteristic of antigen presentation pathways

Thus, the fundamental difference between MHC class II and I peptide processing presentation is therefore the source of peptides and the site of peptide loading. MHC class II presentation presents exogenous foreign (or self) peptides that have been internalised and delivered to the MHC class II loading compartment. Antigens may reach these intracellular compartments following a variety of antigen uptake mechanisms such as phagocytosis, pinocytosis or receptor mediated endocytosis (Watts, 1997). The early endosomes are the first compartment encountered and these have little proteolytic activity in generating peptide antigens for MHC class II loading. The late endosomes and lysosomes provide an acidic pH which permits proteolysis and disulphide bond reduction of polypeptides (Watts, 2001). Acidification is an important factor in protein unfolding and in the activation of proteases. Disulphide bond reduction has more recently been shown to have an important role in the liberation of antigenic peptides (Maric et al., 2001). The endosomal proteases that generate peptides

are classified into families according to the residue that they use to cleave the peptide bond. Most of the lysosomal proteases are known as cathepsins followed by a letter (A, B, C). Studies have shown a great complexity in the activity of many of these proteases in liberating antigenic peptides from precursor polypeptides (Riese and Chapman, 2000).

The co-association of newly synthesised MHC class II-Ii complexes and peptide in the same endocytic vesicle requires a process of Ii chain removal and peptide loading (Denzin and Cresswell, 1995; Sherman et al., 1995). Firstly the Ii chain is cleaved by a variety of cysteine proteases and cathepsins until only the CLIP peptide occupies the binding site (Villadangos et al., 1999). Once the endosomal localisation signal of the invariant chain in removed the MHC class II-CLIP complex can traffic to the cell surface (Bakke and Dobberstein, 1990) . This is not normally seen, implying a substitution of CLIP for antigenic peptide shortly after Ii chain proteolysis. The principle factor that contributes to CLIP removal is the activity of the MHC class II chaperone HLA DM. This heterodimer, which is encoded within the MHC class II region, is non-polymorphic and does not bind MHC class II peptides. It is believed to act upon the CLIP occupied but 'flexible' MHC class II peptide binding groove, to open the groove, and permit CLIP loss. Furthermore, it stabilises the open peptide binding groove providing an opportunity for antigenic peptide binding. This action may take place on other MHC class II loaded complexes whose binding groove is occupied but flexible. This process has been termed kinetic proofreading to convey the functional

context of peptide distinction between optimal and suboptimal peptides (Brocke et al., 2002; Kropshofer et al., 1996). Another MHC lass II chaperone is encountered within the endosomal route. This molecule is HLA-DO and is encoded within the MHC class II region. Like DM it does not bind peptides and has limited polymorphism. It is principally expressed in B cells and its function is not clear. It is thought to promote high affinity peptide binding in late endosomal compartments and support the stabilisation of open MHC class II molecules by HLA-DM (Kropshofer et al., 1999). Once antigenic peptide has bound, the MHC class II molecule is thought to adopt a more rigid conformation which is unsuitable for DM association, permitting egress to the cell surface. Once at the cell surface the MHC class II complexes are internalised for lysosomal destruction or interchange of peptides within the endosomal compartments.

MHC class II presentation ensures that exogenous/internalised proteins are presented to the immune system. This presentation of antigenic ligand and receptor is seen by CD4 T cells. This is undertaken in professional antigen presenting cells for the subsequent activation, proliferation, cytokine liberation and co-stimulatory help that CD4 cells provide to B and CD8 T cells in mediating control of an invading micro-organism.

1.3.3 CD1 ANTIGEN PRESENTATION

Following the advances in understanding the processing and presentation of protein antigens in the late 1980 and early 1990's, a new pathway of T cell antigen recognition was described in 1992 (Porcelli et al., 1992). This pathway was restricted to the presentation of lipid antigens to T cells through a group of molecules encoded outside of the MHC region. This initial description concerned the molecule CD1b which is one of 5 CD1 proteins (CD1a, CD1b, CD1c, CD1d, CD1e) encoded on chromosome 1. The polypeptides that are encoded by the CD1 genes have homology to MHC class I heavy chains containing 3 extracellular domains (α_1, α_2 and α_3). They are also non-covalently associated with β_2-microglobulin (β_2m) (Porcelli and Modlin, 1999). X-ray crystallographic studies of CD1b and CD1d have shown that they have large antigen binding grooves that are lined by hydrophobic amino acids (Gadola et al., 2002; Zeng et al., 1997). These structures show the alkyl chains of the amphipathic lipids interacting with the hydrophobic pocket allowing the hydrophilic component of the antigen to protrude from the groove. Several types of lipid antigen are known to be presented by CD1 molecules including mycobacterial mycolic acids, bacterial glycolipids such as lipoarabinomannan, phosphatidylinositols, sphingolipids, polyisoprenoid lipids and their glycosylated derivatives.

CD1 molecules require an association with the protein folding chaperones calreticulin and calnexin shortly after translation. This ER assisted folding of the glycosylated heavy chain of the CD1 proteins is believed to promote subsequent β_2m and self lipid association. Although the self ligand association has not been conclusively demonstrated, various lines of experimental evidence have supported this concept. In an analogous manner to the role of class II invariant chain (Ii) and peptide (CLIP) in binding to MHC class II molecules (and suboptimal peptide binding to MHC class I molecules), this self ligand association is believed to protect/block the groove until the appropriate ligand is identified (Moody and Porcelli, 2003). Following release from the ER all CD1 molecules are believed to transit to the cell surface before they diversify in their intracellular distribution (Figure 1.3.3).

CD1a molecules are unusual as their cytoplasmic tails lack a recognised internalisation sorting motif. CD1a is therefore found predominantly at the cell surface with a small fraction in sorting endosomes. CD1a has been shown to present glycolipids from mycobacteria and self lipids to $\alpha\beta$ T cells.

CD1b has a cytosolic tail with a tyrosine internalisation sorting motif. This YXXZ (where Z is a hydrophobic residue and X any amino acid) permits an association with adaptor proteins that mediate intracellular sorting. The YQNIP cytoplasmic tail of CD1b permits penetration through to the late endosomes , lysosomes and MHC class II compartments. As CD1b is able to present at least 3 classes of

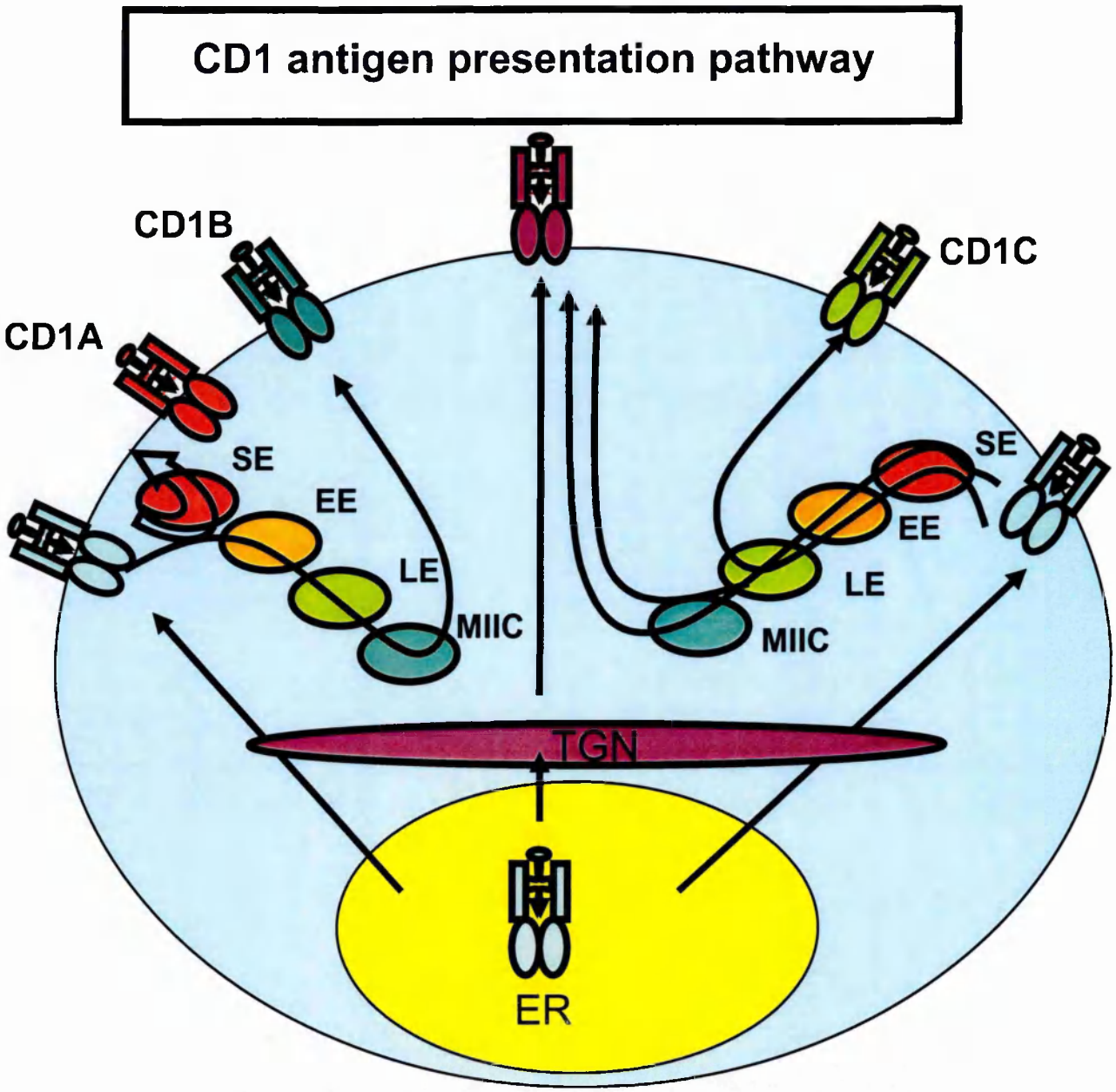


Figure 1.3.3 – CD1 antigen presentation pathway

A schematic representation of the CD1 antigen presentation pathway. The CD1 isoforms traffic through different intracellular compartment from the cell surface. These include the sorting endosome (SE), early and late endosomes (EE/LE) and the MHC class II loading compartment (MIIC). This intracellular trafficking is controlled by cytoplasmic tail interactions with adaptor proteins and the invariant chain association with CD1d.

lipid antigen (mycolates, diacylglycerols and sphingolipids) there may be differing requirements for loading of such lipids within different environments. This has been recently shown for the sphingolipid, ganglioside GM_2, where the length of the alkyl chain of the lipid antigen determined whether it bound to the CD1b molecule within an endosomal ($>C_{40\ long}$) or non-endosomal pathway ($<C_{40\ short}$) (Joyce and Van Kaer, 2003).

CD1c has a YQDI cytosolic motif which permits internalisation through an interaction with the AP-2 adaptor complex but not with the AP3 complex. CD1c is therefore only able to traffic to the early and late endosomal compartments. This surveillance of the endocytic pathway permits loading of CD1c with microbial isoprenoids ligands (Briken et al., 2000).

CD1d is a peculiar CD1 molecule, as it presents an unknown self lipid, has multiple intracellular localisations and selects/activates a particular T cell subset with an invariant $\alpha_{24}\beta_{11}$ gene usage. Its cytoplasmic tail (YQGVL) permits localisation to the early and late endosomes (like CD1c) and a dileucine motif enables sorting to lysosomes (like CD1b). In addition it has been shown to associate with Ii and traffic to the MHC class II compartments (MIIC).

The emergence of the CD1 antigen presentation pathways for the selection and activation of lipid specific T cells complements and extends our understanding of the MHC class I and II pathways for protein antigens. Similarities can be drawn

between the need to initially load a self ligand for correct folding, selective trafficking and the avoidance of degradation. Secondly the capacity of antigen presenting molecules to change their cargo, sample antigens in different cellular compartments and signal to selected effector cells underscores the importance of understanding the molecular nature of these pathways.

1.4 CELL BIOLOGY OF MHC CLASS I ANTIGEN PRESENTATION

1.4.1 PROTEASOME AND ITS SUBSTRATES

The generation of peptides is fundamental to the process of MHC class I presentation. This activity is principally undertaken by the constitutively active peptide producing machinery of the cytosolic proteasome (Kloetzel, 2001). The substrates of the proteasome are usually:

1) Damaged/oxidised proteins at the end of their life

2) Displaced, deglycosylated ER proteins

3) DRiPS (Defective Ribosomal Products)

Such waste disposal of host proteins is required to maintain a homeostasis of new protein synthesis and senescent protein removal. It is this essential housekeeping service that serves as the platform for peptide ligand generation and is necessary to eventually complete MHC class I complex assembly. The constant availability of proteosome/cytsolic peptidase substrate in all cells

provides a continuous source of host peptide to be presented on MHC class I molecules in all nucleated cells. In the presence of foreign proteins, such as those from an invading virus, it would not be acceptable to the host immune system to wait for protein senescence before the foreign proteins can be made into peptides for presentation to the host immune system (Yewdell et al., 1996; Yewdell et al., 2001). This dilemma has been recently addressed by the introducing the concept of protein biosynthesis as an imperfect system with its defective ribosomal products (DRiPs) being channelled through the host peptide producing machinery to yield antigenic peptides. This DRiP hypothesis thus allows the immune system to monitor protein synthesis rates in cells and not protein concentration or degradation. This process of protein substrate generation allows the immune system to sample foreign peptide from the background of self proteins, permitting a rapid detection of infected cells (Yewdell, 2002).

1.4.2 PROTEASOME

The proteasome is a large multicatalytic proteinase that is present in both the nucleus and cytosol of all eukaryotic cells. It is composed of 4 heptameric subunit ($\alpha_7\beta_7\beta_7\alpha_7$) rings which form a hollow barrel with a central chamber in which substrates are degraded (Kloetzel, 2001). This central chamber has the catalytically active faces of the β subunits directed into the central chamber. This catalytic core of the proteasome is known as the 20S proteasome. The other

structural features of proteasome are the regulatory subunits that attach to each end of the 20S proteasome. The major regulatory subunit is the 19S regulator which is able to recognise polyubiquitin chains of protein substrates and direct them into the barrel of the 20s proteasome. The 26S proteasome complex is made from a 19S regulator subunit sited at either end of the 20S core. The base of each regulator subunit is composed of 6 ATPases of the AAA family. This family has an ATP binding site with conserved motifs known as Walker A and Walker B. These are in contact with the 20S catalytic unit resulting in the ATP dependent opening of the α rings, permitting polypeptides access to the proteolytic core. Of the seven β subunits only 3 are catalytically active, β1, β2 and β5, providing 6 active sites within the core. Two cut preferably after hydrophobic residues (β5-'chymotrypsin like'), two cleave after basic residues (β2 –'trypsin like') and the two remaining sites preferentially act after acidic residues (β1 – 'caspase like'). These catalytically active subunits all contain a threonine at their amino termini characterising the proteasome as a member of the amino-terminal nucleophile hydrolase family. In certain tissues these catalytically active subunits may be replaced with interferon γ (IFN- γ) inducible subunits. These inducible subunits are LMP2, LMP 7 and MECL-1. Collectively they are refereed to as the immunosubunits and their incorporation into a new 20S proteasome is identified as the 'immunoproteasome'. A further IFN- γ inducible component of the proteasome is the proteasome activator PA28 (11S regulator). It is composed of 2 IFN inducible subunits PA28α and PA28β. This attaches to the α rings of the 20S proteasome to form a 19S-20S-11S complex. The constitutive

26S proteasome (19S-20S-19S) and immunproteasome 19S-20S (LMP2/LMP7/MECL-1)-11S are believed to maximise the diversity of peptides from precursor polypeptides (Emmerich et al., 2000; Toes et al., 2001). This has been shown at the level of individual peptides where either of these variants may destroy or enhance the index peptide. At the 'population' level of liberated peptides a different repertoire has been identified between constitutive or immunoproteasomal proteolysis (Van den Eynde and Morel, 2001).

The targeting of misfolded, damaged or incomplete proteins to the proteasome is mediated through the ubiquitin pathway. The targeting for degradation is mediated by the attachment of multiple molecules of ubiquitin to the substrate. This small protein covalently attaches to a free amino group on the substrate. The lysine of the preceeding ubiquitin is then targeted by subsequent ubiquitin molecules to generate a polyubuiquinated substrate. This process is undertaken by 3 important enzymes. E1 initiates the cascade through an ATP dependent activation of ubiquitin. This occurs through a thioester bond between the C-terminal carboxyl group of ubiquitin and a cysteine side chain of E1 This is then suitable for transfer to the E2 carrier protein via a trans-esterification reaction to the cysteine residue in the active site of E2. E2 is sufficient to transfer the ubiquitin to the substrate but the third enzyme is usually required to catalyse polyubiquitination. This final enzyme (E3) is ubiquitin protein ligase (E3) and this brings E2 into the vicinity of the substrate in addition to directly catalysing ubiquitin conjugation to the substrate. The polyubiquinated substrate is

recognised by the 19S regulator subunit and fed into the 20 S catalytic core for proteolysis (Kisselev and Goldberg, 2001; Kornitzer and Ciechanover, 2000).

1.4.3 CYTOSOLIC PEPTIDASES

The ubiquitin proteasome pathway is considered to be the major contributor of antigenic peptides for MHC class I assembly. However, the use of selective proteasomal inhibitors has implicated other cytosolic proteases as having a role in antigenic peptide production for MHC class I assembly. Over the last few years a number of these proteases have been identified and studied.

- Tripeptidylpeptidase II (TPII) was described in 1999 as a giant protease that may substitute for some of the proteolytic actions of the proteaosome (Geier et al., 1999). TPII is a ubiquitous serine peptidase that can exist as a cytosolic and membrane bound form. It cleaves aminoterminus tripeptides in a chymotrypsin/trypsin like manner. Additional endopeptidase activity after lysine and arginine residues has also been seen but this is thought to only account for 0.2% of its exopeptidase activity. TPII therefore probably acts to degrade oligopeptides which may be circumvented for MHC class I usage (Wang et al., 2000). Recently this has been identified for the HLA-A3 Nef 73-82 peptide which is reduced when TPII activity is inhibited pharmacologically (AAF-CMK) or by RNA interference (RNAi) (Seifert et al., 2003).
- Bleomycin hydrolase (BH) and Puromycin sensitive aminopeptidase (PSA) were identified in 2000 as having N terminal trimming activities (Stoltze et al.,

2000). BH is a cysteine peptidase (inhibited by E64) that was identified as a cytosolic trimmase of a VSV epitope, whilst PSA is a metalloprotease identified under similar circumstances. Their individual contributions to MHC class I assembly have not been clearly addressed and similar to the proteasome and TPII, both have general housekeeping functions in most cells.

- Leucine aminopeptidase was shown in 1998 to undertake N terminal trimming of an extended peptide to a final MHC class I epitope (Beninga et al., 1998). Furthermore this enzyme was inducible upon IFN-γ treatment, a stimulus for many MHC class I assembly chaperones. Upon inhibition by bestatin this N terminal trimming was lost.
- TOP (thiol-sensitive metallopeptidase) has been most recently shown to behave as a cytosolic endopeptidase. It has been shown to cleave peptides of 6-17 amino acid residues and importantly upon over expression to destroy antigenic peptides (Saric et al., 2001). Manipulation by RNAi showed an improved presentation of antigenic peptides (York et al., 2003) . It can therefore be seen as a limiting factor in the transformation of proteosome derived peptides to MHC class I ligands.

The role of each of these cytosolic peptidases in the preparation of peptides for TAP transport and eventual MHC class I assembly will become clearer over the following years through the selective use of 'knockdowns' and global and selective epitope analysis.

1.4.4 TRANSPORTER ASSOCIATED WITH ANTIGEN PRESENTATION (TAP)

TAP is a member of the ATP binding cassette transporter family (ABC transporters). It is a heterodimer of TAP1 and TAP 2 whose genes are encoded in the MHC class II region of chromosome 6 (Elliott, 1997). The TAP proteins are composed of a hydrophobic domain, comprising of multiple transmembrane segments and a hydrophilic nucleotide binding domain (NBD). The NBD has a highly conserved sequence motif (Walker A and B) that permits ATP binding and hydrolysis. The TAP heterodimer transports peptides from the cytosol to the ER in an ATP-dependent manner (Neefjes et al., 1993b). TAP exhibits some degree of size and sequence specificity for substrate peptides: transported peptides are optimally 9-12 amino acids in size although longer peptides may be transported (Androlewicz and Cresswell, 1996). Recent work using GFP tagged TAP chains, which allows for the calculation of TAP mobility within the ER membrane, has shed new light on the function of TAP. The lateral mobility of TAP in the ER of living cells increases when TAP is inactive and decreases when it translocates peptides (Reits et al., 2000). Using this approach it has been suggested that in 'normal' cells only a third of TAP molecules are actively transporting peptides. However TAP activity is seen to become fully operational following viral infection. The source of extra substrate in these experiments was shown to be defective ribosomal products (DRiP's). The enhanced TAP activity was reduced when the

production of viral DRiP's was impeded. An additional observation was that peptides that could bind to TAP but were not transported could decrease the mobility of TAP. This suggested that a peptide induced conformational change in TAP may occur independently of peptide translocation. This notion has been subsequently supported by experiments showing that peptides or nucleotides can promote different TAP conformations (Karttunen et al., 1999). Using TAP mutants that were unable to bind ATP, Knittler and colleagues found that (Alberts et al., 2001; Knittler et al., 1999);

(i) peptide transport required nucleotide binding to both TAP subunits

(ii) peptide mediated release of MHC class I/β_2m complexes from TAP required nucleotide binding but not hydrolysis (when peptide was exogenously added)

(iii) nucleotide binding was necessary for peptide binding to TAP.

These observations were novel as peptide binding to TAP had previously been considered as a nucleotide independent event and class I release from TAP had not been linked to conformational changes in TAP. They concluded that peptide mediated release of MHC class I/β_2m from TAP is driven by a nucleotide dependent conformational change in TAP. Subsequent work using TAP mutants that would allow ATP binding but not ATP hydrolysis indicated that:

(i) TAP subunits have asymmetrical properties

(ii) TAP1 has a high affinity ATP binding site

(iii) peptide binding and transport requires nucleotide binding of both NBDs

(iv) peptide mediated disassembly depended mainly on nucleotide binding by TAP1

(v) mutations in TAP 2 (but not TAP 1) that permit nucleotide binding but interfere with ATP hydrolysis prevent peptide transport and MHC class I cell surface expression.

Together these studies suggest that the TAP subunits have distinct roles in peptide transport, such that ATP hydrolysis at one chain induces ATP binding at the other and that peptide mediated dissociation of MHC class I from TAP is linked to conformational signals arising from ATP occupation of TAP1. Karttunen and colleagues provided additional evidence for the asymmetrical functions of the TAP heterodimer (Karttunen et al., 2001). They analysed human TAP1 and TAP2 constructs with a point mutation in the Walker binding motif that allows ATP binding but prevents ATP hydrolysis (different mutation to that of Knittler et al). They also identified an enhancement of ATP binding to TAP 1 following nucleotide or peptide binding to binding to TAP2. Furthermore, mutant TAP1/wild type TAP2 permitted peptide binding and transport to support B5 expression but no peptide binding or transport was observed when mutant TAP 2/wild type TAP1 was expressed in TAP mutant T2 cells. It will now be interesting to see how the assembly of TAP associated MHC class I/β_2m complexes with peptide is coupled to this transport cycle and whether this orchestration can improve either the kinetics of peptide acquisition, or the qualitative nature of the loaded peptides.

1.4.5 TAPASIN

In 1994 Greenwood et al described a mutant cell line (721.220) in which TAP was necessary but not sufficient for the stable assembly of MHC class I complexes (Greenwood et al., 1994). A spectrum of allelic differences in the assembly and cell surface expression of differing classical MHC class I complexes was shown to be a post translational event and to follow HC/β_2m assembly. Subsequent work showed that this cell line was defective in a 48kDa protein, previously seen in co-immunoprecipitates of TAP and class I (Ortmann et al., 1994), that bridged MHC class I/β_2m complexes to TAP (Sadasivan et al., 1996). This protein, coined tapasin (TAP –associated glycoprotein), was cloned in 1997 (Ortmann et al., 1997), and shown to reconstitute normal MHC class I assembly in 721.220. Tapasin is an ER resident type I transmembrane protein that is encoded by a gene within the extended MHC class II region of chromosome 6 in humans. The genomic structure of tapasin is seen in Figure 1.4.5.1. The coding sequence of the gene spans 12 357 bp of DNA situated 200 KB centromeric of the DP locus. It contains 8 exons, a promoter with an IFN-γ response element and 2 very large introns situated between exons 3/4 and 7/8. Exon 1 (52 bp) and exon 2 (171 bp) contain the leader sequence and N terminus of the mature protein. Exon 3 (261 bp) encodes a region that has sequence homology to a partial immunoglobulin superfamily (Ig) domain. Exon 4 (399 bp) has no homology to other sequences (Exon 4 of chicken tapasin does have homology to to other sequences) and contains a dimorphism, whilst exon 5 (342

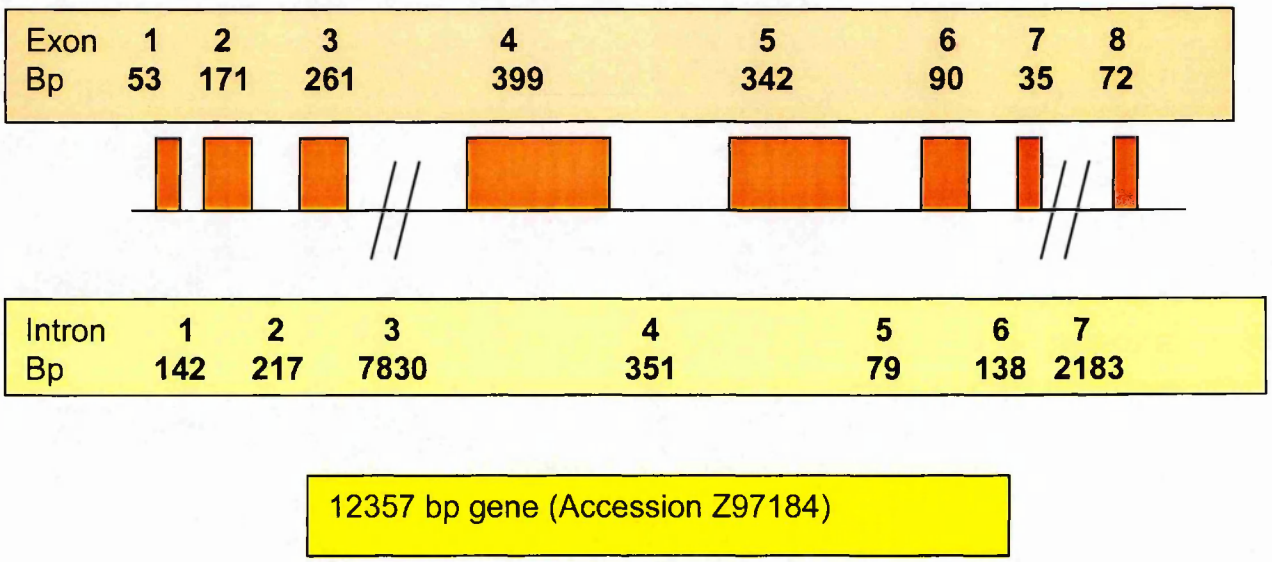


Figure 1.4.5.1 –Tapasin gene structure

The human tapasin gene is approximately 13,000bp with 8 exons. Exons 2, 3 and 4 encode 75% of the mature protein. There are 2 large introns (>2000bp) and a 5' sequence that possess a IFN-γ response element. The mouse tapasin shares a similar genomic structure within the murine H-2 locus.

bp) contains a domain with homology to a complete Ig domain. .Exon 6 (90 bp) contains the connecting peptide and part of the transmembrane region whilst exon 7 (35 bp) and exon 8 (72 bp) form the remainder of the transmembrane region and the cytoplasmic tail. Klein recently suggested, using sequence structure predictive algorithm programming that tapasin had the greatest structural association with the Ig C1 domain of the MHC class I heavy chain (Mayer and Klein, 2001). Furthermore this matching was greatest for amino acid residues encoded in exon 5. Other strong matches were found for the first two β strands of the class I α domain and exon3 and the homologous part of the $\alpha 2$ domain and exon 4. No profile could be identified for exon 2 and the first half of exon 3. A schematic of the human tapasin and MHC class I gene organisation is seen in Figure 1.4.5.2. The mature tapasin protein is 428 amino acids and migrates as a 48 kDa band by SDS-PAGE electrophoresis. It has a single glycosylation site and an ER retention/retrieval sequence (Herberg et al., 1998b). Subsequent investigations have suggested that tapasin is involved in many aspects of MHC class I assembly (Momburg and Tan, 2002). In addition to bridging MHC/β_2m heterodimers to TAP, it increase TAP levels, promotes peptide binding by TAP and facilitates the incorporation of other chaperones in to a multi-molecular peptide loading complex (PLC) at the TAP interface. The molecular mechanisms mediating these multiple functions are poorly understood at present and are investigated within this thesis.

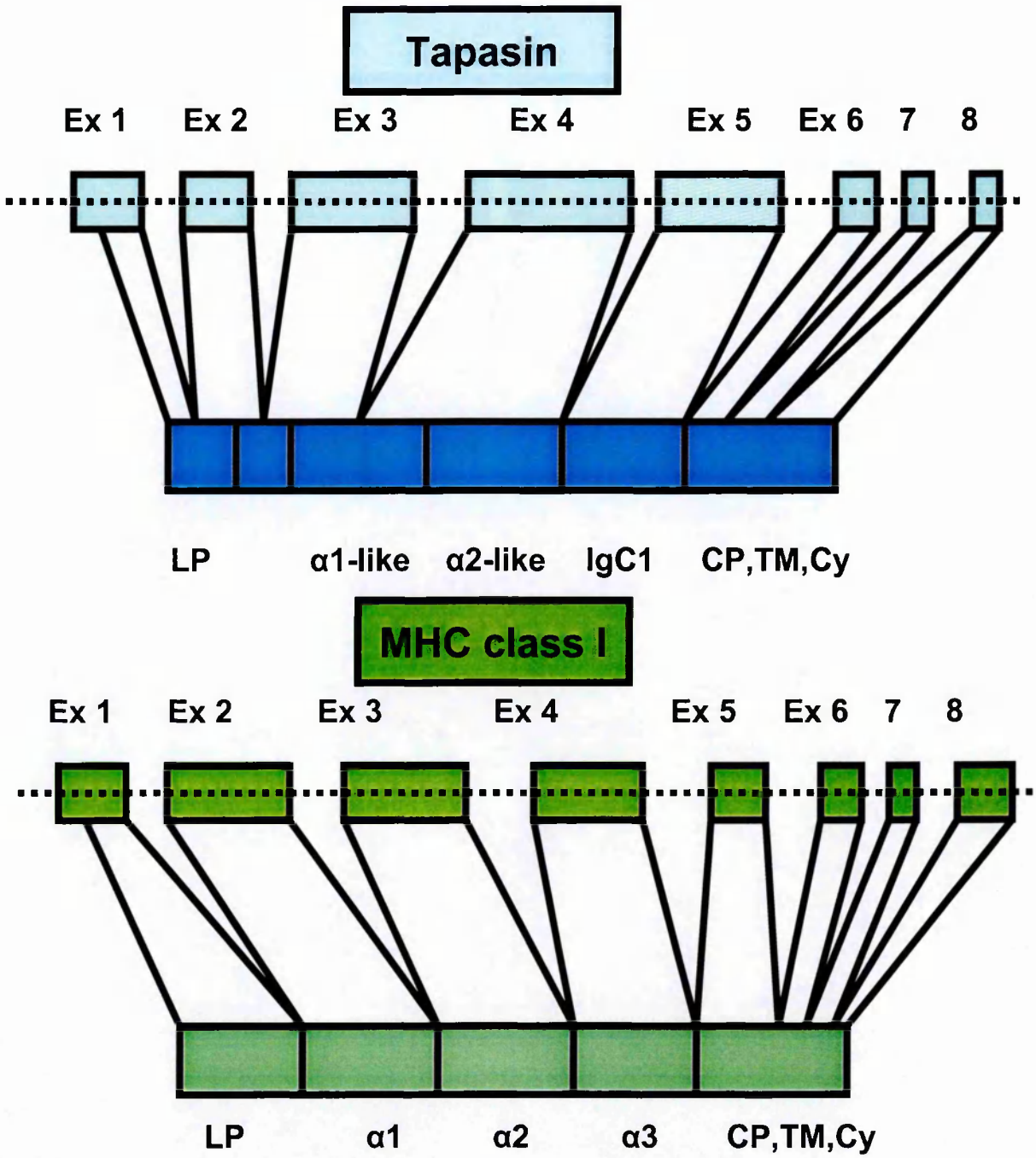


Figure 1.4.5.2- Comparison of the gene organisation between tapasin and the MHC class I HC.

The protein domains are LP (leader peptide), α1 and α2 peptide binding domains. IgC1 is the immunoglobulin C1 domain and CP (connecting peptide), TM (transmembrane), Cy cytoplasmic domain.

1.4.6 CALRETICULIN

Calnexin and calreticulin are glycoprotein specific chaperones that assist in the folding of newly synthesized glycoproteins in the ER (Parodi, 2000). Calnexin was first described as glycoprotein-specific ER protein in 1993, possessing an ER localisation sequence at its C terminus (Bergeron et al., 1994). Calreticulin is a glycoprotein specific chaperone that assists in the folding of newly synthesised glycoproteins in the ER (Johnson et al., 2001). It is a soluble protein possessing a KDEL ER retrieval sequence at its C terminus, a proline rich central P domain and both low affinity and high affinity calcium binding sites. Its principal interactions are with newly synthesised glycoproteins that bear high mannose glycans with a single terminal glucose residue arising from either trimming of the tri-glucose extension by the action of ER glucosidase 1 and 2 or through the reglucosylation of the man^9 core via the action of UGGT (Helenius and Aebi, 2001). Calreticulin (together with calnexin) undertakes a process of ER glycoprotein quality control that ensures that only correctly folded and assembled glycoproteins leave the ER. This calnexin/calreticulin quality control mechanism, in association with ERp57, acts upon monoglucosylated glycoproteins to promote their folding and disulphide bond formation (High et al., 2000). Dissociation of a glycoprotein from calreticulin exposes the terminal glucose to trimming by ER glucosidase 2, and the option to enter the secretory pathway. However, if is the glycoprotein is incompletely folded, it is rapidly reglucosylated by UDP-glucose

glucosyltransferase (UGGT) allowing the protein to undergo another cycle of glycoprotein chaperone association (Ellgaard and Helenius, 2003). Proteins that are persistently misfolded are retained in the ER prior to degradation. This degradation process is termed ERAD (ER associated degradation) and promoted by mannose trimming of the oligosaccharide unit by ER mannosidases I and II (Tsai et al., 2002). The individual contributions of calnexin and calreticulin to MHC class I assembly have been facilitated through studies on knock out cell lines. A mouse calreticulin KO cell line study has shown an important contribution of calreticulin in MHC class I assembly (Gao et al., 2002). The absence of calreticulin leading to an 80% reduction in total cell surface MHC class I, a failure to generate specific MHC class I peptide complexes and a change in the trafficking rate of MHC class I molecules. The exact molecular mechanisms mediating this altered function are currently unclear and similar studies are awaited for human calreticulin

1.4.7 CALNEXIN

Calnexin is an ER transmembrane bound lectin that has specificity for newly synthesised glycoproteins with a single glucose residue. It is the first glycoprotein to associate with MHC class I heavy chain (Degen and Williams, 1991; Jackson et al., 1994; Paulsson et al., 2001a). However, studies of a human calnexin deficient cell line have shown no obvious reduction in class I presentation at the cell surface (Scott and Dawson, 1995). Nevertheless, upon

correct folding and β_2M association human HC complexes normally dissociate from calnexin whilst mouse complexes remain associated until peptide is acquired in the PLC (Harris et al., 1998). Calnexin has been shown to be associated with TAP and tapasin in human cell lines and this complex has been termed the pre PLC (Diedrich et al., 2001). Following HC:β_2M incorporation, calnexin is thought to dissociate from such a complex. The purpose of such a pre-PLC is not understood at present. Calnexin has an important role in the removal of glycoproteins from the ER if they have failed to fold correctly. This process involves calreticulin within a calnexin/calreticulin quality control pathway (Chevet et al., 2001). Upon correct folding of protein the terminal glucose is trimmed by ER glucosidase II leading to a failure to re-associate with calnexin. The glycoprotein may than leave the ER or if identified as incompletely folded by the glycoprotein 'sensor' UGGT it may undergo a further reglucosylation for another round of calnexin associated folding. Recently another pathway that is competing with this pathway has been identified (Molinari et al., 2003; Oda et al., 2003). The trimming of the Man_9 backbone to Man_8 by ER mannosidase I lowers the affinity of the glycoprotein for UGGT mediated reglucosylation. Additionally this oligosaccharide becomes a ligand for a recently described ER receptor termed EDEM (ER degradation-enhancing 1, 2 mannosidase-like-protein). This glycan signal recognition factor is associated with calnexin but requires release of substrates from calnexin (through deglucosylation) to mediate enhanced glycoprotein degradation. Finally it appears that retrotranslocation to the cytosol for proteasomal mediated removal of such misfolded glycoproteins is required to

complete the degradative process. How the calnexin/EDEM complex co-operates with the Sec 61 translocon is currently not known.

1.4.8 ERP-57

This protein is the most recent component to have been identified as being important in MHC class I assembly. In 1998, 3 groups identified ERp57as a component of the PLC (Hughes and Cresswell, 1998; Lindquist et al., 1998; Morrice and Powis, 1998). In contrast to TAP and tapasin an ERp57 knockout cell line does not exist and therefore information regarding its function is currently inferred from consideration of other mutant cell lines. ERp57 is a thiol dependent oxido reductase (containing 2 thioredoxin motifs) that has been shown to mediate disulphide bond formation in monoglucosylated glycoproteins, often in association with the lectin binding chaperones calnexin/calreticulin. The association of ERp57 with a target glycoprotein is dramatically reduced if the glycan trimming to a monoglucosylated form is prevented (Elliott et al., 1997; Farmery et al., 2000; Oliver et al., 1997). ERp57 may also function as a more general chaperone as it has been found to associate with non-cysteine containing glycoproteins and is glucose regulated like the classical molecular chaperones BiP and GRP94. Thus ERp57 may operate as a 'general chaperone' as well as having a more specific role in disulphide bond formation. Within the PLC the function of ERP57 is not known. The peptide/HC/β_2m complex has a glycosylation site in the alpha 1 region and cysteines at position

101 and 164 in the alpha 2 domain and 203 and 259 in the alpha 3 domain of the HC. These are fully oxidised when the peptide/HC/β_2m complex reaches the PLC (Dick et al., 2002). In studies of the tapasin deficient cell line 721.220, ERp57 does not associate with the TAP complex. Experiments in the β_2m negative Daudi cell where no HC/β_2m complex is seen within the PLC could identify ERp57 in association with TAP/tapasin and calnexin (Diedrich et al., 2001). ERp57 has been identified in the PLC of calreticulin deficient cell lines (Gao et al., 2002). Furthermore in TAP deficient cells, ERp57 has been identified in a tapasin/CRT/ HC/β_2m subcomplex (Hughes and Cresswell, 1998). Therefore it appears that the incorporation of ERp57 into the PLC is only critically dependent upon tapasin and independent of the HC/β_2m complex or calreticulin. Studies of cells transfected with N terminal deletion tapasin constructs, that do not bridge HC/β_2m complexes to TAP, fail to associate ERp57 with TAP. This was observed when only the N terminal 50 amino acids were absent from the 428 amino acid tapasin protein suggesting that this region is important for an interaction with tapasin or that deletion of this region disrupts the structure of tapasin and accordingly the ERp57 association (Bangia et al., 1999). We await further studies on the role of ERp57 within the PLC and additional techniques to delineate the function of ERp57 within the both early and late phases of MHC class I assembly.

1.4.9 ENDOPLASMIC RETICULUM (ER) PEPTIDASES

The ability of N terminally extended peptides to be delivered into the ER and be correctly presented suggested that aminopeptidase activity within the ER could occur (Elliott et al., 1995; Snyder et al., 1994). The identity of such a ER aminopeptidase remained elusive until 2002 when two groups identified such an aminopeptidase (Saric et al., 2002; York et al., 2002). Goldberg and colleagues identified ER-aminopeptidase I (ERAP1) as an interferon inducible aminopeptidase that was inhibited by the drugs leucinethiol and 1,10-phenanthraline. Overexpression of this aminopeptidase increased the cell surface expression of an N terminally extended model peptide, suggesting that in vivo trimming is rate limiting for antigen presentation. Further studies suggested that ERAP1 could also destroy peptides in the ER, limiting antigen presentation at a bulk level. Shastri and colleagues showed that ERAP (termed ER aminopeptidase associated with antigen presentation [ERAAP]) failed to cleave the X-Pro bond which they had previously established as classical for this leucinethiol inhibited ER aminopeptidase. Furthermore they showed that ERAP's proteolytic activity is controlled by the length of the peptides substrate becoming less active on 8-mer and 9-mer peptides which are optimal for MHC class I loading.

In contrast to the aminopeptidase activity that has been established within the ER, there appears to be little evidence for carboxypeptidase activity (Lobigs et al., 2000; Snyder et al., 1997). The other recognised peptidase activity that has

been shown to influence MHC class I assembly within the ER relates to signal peptidase (SP) and signal sequence peptidase (SPP) (Martoglio and Dobberstein, 1998). The first of these is known to trim the signal sequence from ER translocated proteins and was initially shown to be important in providing hydrophobic peptides in a TAP independent manner from alleles such as HLA A2 (Henderson et al., 1992). It has also be shown to be important from providing peptides from the signal sequences of MHC class I alleles for subsequent loading upon the non classical allele HLA-E (Lemberg et al., 2001) . Lastly SPP has recently been shown to undertake an important intramembrane cleavage step in liberating signal peptides for subsequent HLA-E binding (Bland et al., 2003).

The ER mediated trimming of peptides completes the proteolytic cascade which begins with cytosolic multiunit proteases such as the proteasome and TPII, through specific cytosolic peptidases, TAP transport and final N terminal trimming by ERAP and perhaps other ER resident trimmases.

1.4.10 MHC CLASS I

1.4.10.1 Structure and function

MHC class I complexes are a trimeric association of MHC class I heavy chain, β_2M and peptide. The MHC class I heavy chain associates with the β_2M subunit to a form a peptide receptive heterodimeric complex. The structure of this

peptide receptive heterodimeric protein has proved difficult to analyse as it is believed to be of a molten globular form and thus resists crystalisation approaches. Important biochemical observations about its structure were initially made by Bouvier and Wiley (Bouvier and Wiley, 1998). Thermolytic digestion of peptide vacant and occupied complexes revealed cleavage sites in the α_2 and α_3 helices identified at temperatures between 15-37°C for the unoccupied complex and above 60°C for peptide occupied complexes. Utilising the hydrophobic binding dye ANS which is used to probe proteins in a molten globule state, they demonstrated increased binding to the complexes in the peptide unoccupied state. Studies by Schneck and colleagues utilised fluorescence energy transfer (FRET) to examine the structure of peptide occupied and vacant complexes (Catipovic et al., 1994). A fluorescently labelled antibody to the α_2 domain of the MHC class I HC and one to β_2M were used for FRET analysis. In the peptide vacant state no FRET was observed, however upon peptide binding FRET was detected suggesting a change in conformation from that of an extended configuration to a more compact form. Furthermore the degree of FRET was influenced by the bound peptide suggesting further conformer variation within the peptide occupied form. Functional analyses of peptide receptive murine D^b complexes by Springer et al extended these observations and demonstrated, through tryptophan fluorescence studies, real time binding of peptide to peptide receptive complexes (PR) (Springer et al., 1998). Furthermore the calculations of association rates exceeded those predicted by equilibrium constants and dissociation rates, suggesting that peptide binding to PR MHC class I complexes

is not a single step association/dissociation. It is suggested that part of the binding energy is used to allow a conformation change to occur in the molecule. This extended previous data on D^b in cell lysates of peptide deficient cells, where conformational changes in the D^b complexes were monitored by conformational dependent antibodies upon peptide and β_2M addition (Townsend et al., 1990). The crystal structure of a D^b peptide occupied complex showed that approximately three quarters of the peptide was buried within the binding groove, suggesting that this assembly must initially take place within a MHC class I complex bearing an 'open groove ' around which a conformational change may take place (Fremont et al., 1992).

The peptide occupied complex has a cooperative stability contributed by both peptide and β_2M (Elliott, 1991). Among different MHC class I heavy chains notable differences exist in the affinity of the HC for β_2M, with the mouse allele L^D have a well documented low affinity which may contribute some of its unique presentation functions (Hansen et al., 2000). Within a single allele the cooperative stability is influenced by the bound peptide leading to the concept of high affinity, high stability long lived complexes and lowered affinity, less stable peptide occupied complexes. This is best demonstrated for the mouse allele D^b expressed in the peptide transporter incompetent cell line RMA-S. Originally this allele was thought to be devoid of peptide as indicated by its thermo-instability as compared to D^b expressed in the normal cell line RMA (Ljunggren et al., 1990). Recently, peptide elution analysis at reduced temperatures has enabled the isolation of peptides from D^b expressed in RMA-S cells (De Silva et al., 1999).

Figure 1.4.10.1a– Structure of a peptide occupied MHC class I complex and with a T cell receptor

The human A6 TCR is seen associated with HLA A2 complexed with the HTLV-1 TAX peptide P6a.
The MHC class I heavy chain is coloured cyan, the β2M is magenta and the TCR α and β chains blue and pink. The image was generated through the Protein Explorer Front door program via accession number 1QRN

Figure 1.4.10.1b – Structure of a peptide occupied MHC class I complex and with a NK cell receptor

The co-crystal structure of the NK cell receptor KIR2DL2 and the MHC ligand MHC-Cw3 with the peptide GAVDPLLAL. The MHC class I heavy chain is cyan, the β2M magenta and the KIR2DL2 blue. The image was generated through the Protein Explorer Front door program via accession number 1EFX .

This suggests that low affinity peptides normally occupy these complexes in comparison to high affinity peptides within thermostable complexes in competent cell lines. The choice of peptide not only determines the stability of a complex but also provides the unique ligand for TCR recognition. Within every MHC class I bound peptide complex a signature is created of the peptide side chains that extend out of the groove within the context of the α_1 and α_2 helices of the MHC class I HC. These form the contact points for the TCR and direct the specificity of the MHC class I ligand TCR interaction (Figure 1.4.10.1a). With regard to NK cell receptor recognition, seminal studies on KIR and HLA-C allele association have shown a distinct but overlapping footprint to that of the TCR (Figure 1.4.10.1b). The KIR (KIR 2DL2) appear to contact the α_3 helix and have a peptide contribution restricted to residues P7 and P8.

The purpose of such presentation in the context of T cells is the transmission of a message from the cell with regard to its internal protein array. In the setting of an infection, the novel peptide MHC class I complexes may activate the T cell to divide and differentiate in to a cytotoxic CD8 T cell. For NK cells the self peptide MHC class I complexes are believed to present a signal of cellular 'well being' transmitted as a negative signal for the NK cell's default killing program.

Finally it should be remembered that the peptide selection of host proteins early in the ontogeny of the immune system allows for the T cell selection of self. This critical role of the self peptide MHC class I complex for CD8 T cell development is elegantly seen in human and mouse models of TAP deficiency, where CD8 cells are absent ((Gadola et al., 2000; Van Kaer et al., 1992).

1.4.10.2 Peptide occupancy and vacancy

The conventional view of MHC class I complex occupancy is one of two forms, peptide occupied and peptide vacant. In contrast, MHC class II molecules are considered as empty, low affinity ligand and high affinity ligand bound complexes (Sadegh-Nasseri et al., 1994; Zarutskie et al., 2001). Furthermore the empty MHC class II complex has been considered as existing in at least 2 forms (Rabinowitz et al., 1998). The first of these isomers is believed to be peptide receptive and have a short half life prior to conversion to isomer 2 which resistant to peptide binding. The low affinity ligand bound complex is the CLIP MHC class II complex that forms in the ER. The suggested roles of CLIP are diverse including directing secretory traffic to the endosomes, preventing empty MHC class II complex aggregation and promoting isomer 1 formation following CLIP removal. The high affinity ligand complex is also believed to exist in more than one conformation as suggested by kinetic studies on single peptide species class II complexes (Sadegh-Nasseri and Germain, 1991). It is believed that high affinity peptide occupied complexes convert to a long lived high affinity peptide complex over time. This may involve reconfiguration of the peptide within the binding groove until the optimal side chain interactions and hydrogen bound networks have been established (Belmares et al., 2003; Schmitt et al., 1999). In biochemical analyses the distinction between empty/low affinity complexes and high affinity complexes has been their susceptibility to SDS mediated subunit

MHC class II occupancy and vacancy

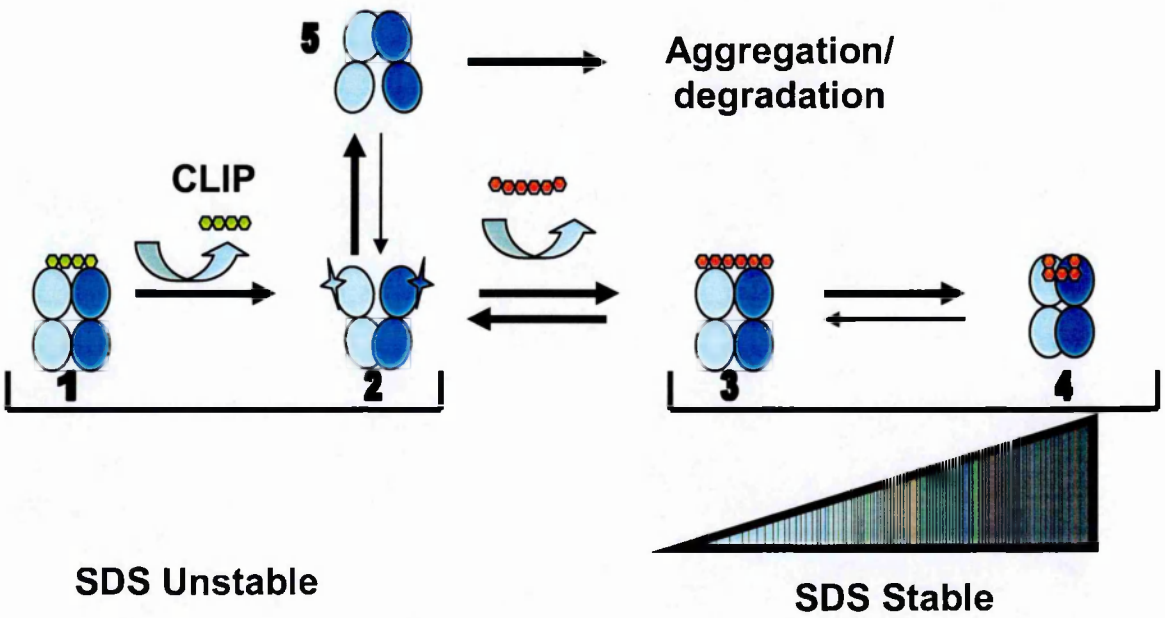


Figure 1.4.10.2 - Schematic representation of MHC class II isoforms

Complex 1 is the CLIP occupied SDS unstable complex that is the precursor of a peptide receptive MHC class II complex (2). This complex has a short half defaulting to a complex that is peptide non-receptive and prone to aggregation (5). HLA-DM is believed stabilise complex 2 and possibly promote its generation from complex 5. Optimal peptide may then be loaded to become complex 3. DM may further act on this peptide loaded complex, promoting isomerisation of the peptide occupied complex so that it may become a long lived complex (5).

dissociation (Chou and Sadegh-Nasseri, 2000; Denzin and Cresswell, 1995; Natarajan et al., 1999) . The concepts and experimental observations of the above groups have been tentatively collated into the Figure 1.4.10.2.

As mentioned earlier there is no substantive evidence to suggest that MHC class molecules behave in an analogous manner. Recent studies by Hansen and colleagues have described an antibody (64-3-7) which recognises non peptide occupied complexes (Yu et al., 1999). Their studies have shown that some intracellular 64-3-7 complexes in tapasin knock out cell lines fail to load exogenous peptide suggesting that at least 2 forms of empty complex may exist (Myers et al., 2000). Studies by Springer et al also suggested an early empty complex which they termed an encounter complex. This was proposed to form an intermediate with peptide which could proceed to a more permanent peptide occupied complex following isomerisation (Springer et al., 1998). The concept of a suboptimally occupied or low affinity ligand occupied MHC class I complex was initially suggested by studies on the MHC class I mutant A2 molecule T134K (Lewis and Elliott, 1998). The key observation in this study was that class I complexes do become occupied with suboptimal ligands even in the presence of a normal peptide supply and that these complexes may egress the ER. It is currently presumed that the inability of these mutant complexes to interact with the PLC contributes to this phenotype implying that such an interaction normally has a qualitative effect upon such low affinity complexes. In a similar manner, MHC class II complexes that fail to interact with DM continue to be occupied with their low affinity ligand CLIP. With regard to high affinity loaded complexes, no

counterpart to the MHC class II single peptide occupied complex that becomes more stable over time has been definitively identified.

1.4.10.3 Export and secretory pathways

The secretory pathway allows cells to regulate the delivery of proteins, carbohydrates and lipids to the plasma membrane and other sites. It consists of a series of distinct organelles and tubulovesicular transport proteins that support transmembrane cargo flux (Lippincott-Schwartz et al., 2000). Within such membranes, posttranslational processing such as proteolysis and glycan modification occurs, which can identify and modify the transport routes chosen by such proteins. The ER is the starting point of the secretory pathway with the cargo packaged for delivery to the plasma membrane, nucleus or other end point. For MHC class I molecules that are destined to reach the plasma membrane, the pathway involves passage through the golgi stacks prior to post golgi trafficking to the cell membrane. The newly synthesised proteins within the ER are required to undergo correct folding/unfolding reactions prior to the export from the ER. In the case of some glycoproteins this involves sequential glycosylation/reglycosylation cycles, mediated through glycoprotein folding sensors within the secretory pathway (Zuber et al., 2001). In order to prevent the default export of soluble and membrane spanning proteins a series of molecular mechanisms are employed to retain and retrieve proteins that are not competent for export. Retention relates to the active process of maintaining a protein within

the ER through immobilisation or extensive interaction with ER resident chaperones. Retrieval relates to the ability to selectively return proteins to the ER that may have escaped initially the ER but are identified as incompletely folded within the early secretory pathway (Vashist et al., 2001). This latter mechanism is best understood through the experimental work undertaken on cytoplasmic tail motif KDEL of certain proteins. This is recognised as a ligand for a KDEL receptor within the secretory pathway which in turn is coupled to a retrograde secretory complex that can return KDEL proteins and their cargo back to the ER (Aoe et al., 1998; Yamamoto et al., 2001). Within the MHC pathway both calreticulin and ERp57 bear such motifs and are resident ER members. Another well recognised retrieval motif is a dilysine motif within the cytoplasmic tail. Tapasin posses such a tail and it has been postulated that this is both active and important in MHC class I assembly.

Following their correct assembly, proteins destined for export from the ER are concentrated at ER exit sites. These are organised membrane domains adjacent to tubule clusters called pre golgi intermediates (Donaldson and Lippincott-Schwartz, 2000). The partitioning into ER exit sites in mediated by the activity of a ras related GTPase (Sar1) and a cytosolic protein complex known as COPII. The COP II complex assembles onto ER exit site membranes following GDT>GTP exchange upon Sar1. Subsequently Sec23/24 and Sec 13/31 heterodimers are assembled on to the membrane to form a COPII bud. This is then believed to form a vesicle for subsequent tubulovesicular mediated egress (Antonny and Schekman, 2001) . The egress to pre-golgi intermediate

compartments allows a further opportunity for sorting and possible recycling through retrograde transport back to the ER. This has been most readily seen for the lectin like protein ERGIC 53 which constitutively recycles between the ER and golgi (Hauri et al., 2000). Its retrograde transport is microtubule dependent being inhibited at low temperature (15°C) and in the presence of microtubular toxins (i.e. nocodozole). The retrograde transport of proteins is thought to occur through the action of another coatmer complex known as COPI. This heptameric cytosolic complex assembles on golgi membranes and this is dependent upon the presence a GTP binding protein ARF1 and GTP. For K(X)KXX or K(X)KXX-proteins this motif binds directly to the COPI coatomer complex (Wieland and Harter, 1999). For KDEL tagged proteins a KDEL receptor may engage this retrograde pathway. Studies of the non classical molecule HLA-G, which possesses a dilysine, have demonstrated COPI associated recycling (Park et al., 2001). Studies on classical class I molecules have failed to show a retrograde transport route apart from an early report in a TAP incompetent cell line (Hsu et al., 1991). This demonstrated retrograde transport of a mouse allele which was not apparent at steady state. Such recycling has also been indirectly suggested for other class I molecules that show faster trafficking in calreticulin deficient cells (failure of KDEL) (Gao et al., 2002) and for tapasin associated complexes in the PLC (KK mutants) (Paulsson et al., 2002).

Upon reaching the trans-golgi, anterograde cargo is packaged into post-Golgi carriers that are within large pleomorphic tubular supports. Most studies have utilised GFP tagged proteins to follow these latter pathways, with little information

available for MHC class I specific pathways. Some studies have indicated that MHC class I complexes may exit the golgi and not directly traffic to the cell surface (Joyce, 1997). Furthermore an absence CD99 has been shown to coincide with such a reduced delivery of MHC class I complexes to the cell surface despite normal medial golgi passage (Sohn et al., 2001). Further research into these areas is needed to clarify these later anterograde pathways

Upon reaching the cell surface the MHC class I complexes can undergo 2 further partitions. Preliminary studies suggest that in a similar way to MHC class II complexes, MHC class I complexes may cluster within lipid rafts to mediate effective signalling (Vogt et al., 2002). The other trafficking opportunity for cell surface complexes is cell membrane to endosome transportation (endocytosis). This process may be undertaken by clathrin dependent or independent mechanisms. Clathrin-dependent internalisation involves the assembly of clathrin and its associated adaptor protein (AP-2) on the plasma membrane (Takei and Haucke, 2001). The membrane protein them becomes part of the budding coated pit of the membrane and internalised. Small GTPases have been implicated in the regulation of such recycling. One such GTPase, dynamin has been shown to be important for MHC class II internalisation (Wang et al., 1997). The ADP-ribosylation factor family of proteins (ARF) are another group of small GTPases that are thought to regulate this endocytosis. ARF-6 has been shown to be important for clathrin-independent MHC class I internalisation through studies of expressed dominant mutants of this protein (Radhakrishna

and Donaldson, 1997). Indeed, the utilisation of this pathway by the HIV protein *Nef* (elegantly worked out by Blagoveshchenskaya and colleagues) is suggested to contribute to the downregulation of MHC class I complexes in early HIV infection, contributing to the impairment of T cell control of the virus (Blagoveshchenskaya et al., 2002). Lastly a particular tubular protein EHD-1 has been shown to be important for clathrin-independent MHC class I internalisation and to colocalise with ARF-6 within this recycling network. This protein appears to associate with ARF-6 containing tubules allowing MHC class I to normally traffic between the cell surface and endosomes (Caplan et al., 2002).

This process of internalisation from the cell surface to the endosome may be functionally important. Several studies have shown that this permits an exchange of peptides within the acidic endosomes (Kleijmeer et al., 2001; Schirmbeck and Reimann, 1996) and is one form of TAP independent cross presentation of antigen. Other studies have suggested that such a pathway is important for the derivation of MHC class I homodimers which may have physiological or pathological functions (Bird et al., 2003). Such recycling may also refine the peptide repertoire by destabilising certain pH sensitive peptide cargos and preventing these from returning to the cell surface (Chefalo and Harding, 2001). Lastly it should be mentioned that certain MHC class I may be secreted from the cell as a transmembrane lacking soluble protein through exon splicing. This has been particularly shown for the non classical allele HLA-G (Lee et al., 1995). MHC class I complexes may also be acted upon by cell surface trimmases that target β_2M dissociated complexes to liberate a 39 kDa heavy

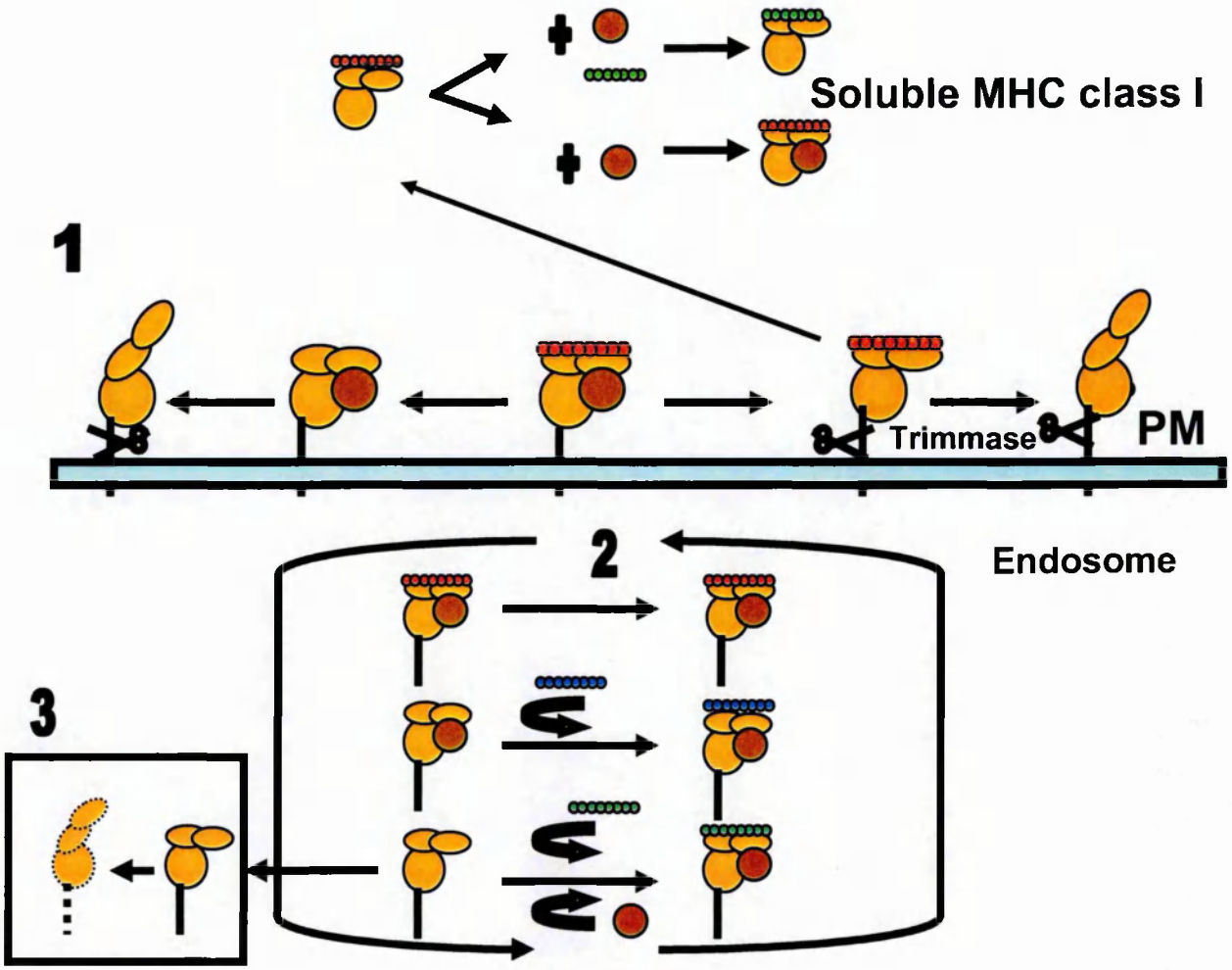


Figure 1.4.10.3-Schematic representation of post golgi trafficking of MHC class I complexes

After leaving the ER-golgi network the MHC class I complexes arrive at the cell surface. They may then enter an endosomal transport system that delivers them into endosomal/lysomal vesicles. This may promote peptide exchange (2) through the liberation of peptide sensitive peptides or lead to HC degradation following β_2M dissociation (3). Alternatively the complexes may transit between the cell surface and endosomal compartments unchanged. MHC class I complexes may also end their lifespan through the liberation at the cell surface of free heavy chain following the loss of peptide or β2M (1).

chain which may re-associate with extracellular peptide and β_2M to form a 37 kDa complex (Demaria and Bushkin, 2000);(Demaria et al., 1999) . The physiological significance of these soluble MHC class I complexes is not known but they have been implicated in autoimmune diseases and transplantation settings ((McDonald and Adamashvili, 1998)(Figure 1.4.10.3).

1.5 INTRODUCTION TO THE PROJECT

My thesis examines the genetic and functional properties of tapasin within the context of MHC Class I complex assembly. I set out to define the degree of polymorphisms in the tapasin gene and the relevance of this to its role in MHC class I peptide assembly. I have developed an in vitro assay to explore the function of tapasin in optimising the peptide selection of MHC class I complexes. I will present the experimental observations of this work and the integrate the findings into a novel working framework for exploring the molecular mechanisms of MHC class I peptide selection and assembly

CHAPTER 2 MATERIALS AND METHODS

2.1. GENERAL TISSUE CULTURE AND CELL LINES

All cells were grown at 37°C within an atmosphere of 5% CO_2 air. All tissue culture media was obtained from Gibco BRL, UK. Foetal calf serum (FCS) was supplied by M.B. Meldrum Ltd UK. All cells were grown in RPMI 1640 media supplemented with 10% FCS, 50 U/ml Penicillin, 50 μg/ml Streptomycin and 2mm L-Glutamine [R10]. All disposable tissue culture material was supplied by Falcon (Becton Dickinson, USA). Cell lines were frozen in 20% DMSO/80% FCS for 24 hours within a Nalgene Cryo 1°C freezing container prior to storage in liquid nitrogen.

721.220 cell are human EBV transformed B cells that are deficient in tapasin function. They are derived from an original EBV transformed B cell line known as LCL 721 (DeMars et al., 1985). This cell line has undergone further gamma irradiation and anti class I complement mediated selection to provide a range of mutant cell lines with interesting MHC class I and II assembly properties. 721.220 is hemizygous across the MHC class I and extended class II region. It has retained expression of the MHC class I allele Cw1 and the class II DR and DQ alleles. It has been shown to carry a point mutation in the acceptor splice site of intron 2 (Copeman et al., 1998). This leads to the aberrant splicing out of

exon 2 which encodes the leader sequence of the protein. The protein is not efficiently translated and is non functional.

721.220.B*4402, 721.220.B2705 and 721.220.B*4405 are single allele transfectants of the tapasin deficient 721.220 founder cell. The cell lines 721.220.B*4402.Tapasin, 721.220.B*4402.Tapasin, 721.220.B*2705.Tapasin and 721.220.B*4405.Tapasin are immunocompetent partner cell lines that express a functional tapasin protein. 721.220.B*4402.Tapasin.ICP47, 721.220.B*2705.Tapasin.ICP47 and are cell lines that are incompetent for TAP mediated translocation of peptides. 721.220.B*4402.ICP47, 721.220.B2705.ICP47 are deficient in tapasin and TAP function. Finally, 721.220.B*4402.Sol Tapasin, 721.220.B2705.Sol Tapasin and 721.220.B*4405.Sol Tapasin are cell lines that have been reconstituted with a soluble tapasin protein that does not associate with the TAP complex.

2.2. Antibodies

The following antibodies were used for immunoprecipitation, flow cytometry, cell selection and western blotting.

W6/32 – a mouse monoclonal antibody that recognises a conformational epitope within a folded β_2M/MHC class I heavy chain complex (Parham et al., 1979). For immunoprecipitation studies it was used at a final concentration 10 μg/ml and for flow cytometry at 1 μg/ml.

116.5.28 – a mouse monoclonal antibody that recognises the Bw4 epitope on B*4402 and B*2705 (Saxon Europe,UK)

RING 4C –a rabbit polyclonal antibody against human the cytoplasmic tail of TAP2 (a gift from P Cresswell, Yale University school of Medicine, New Haven.). Used at 10 μg/ml for immunoprecipitations and 1 μg/ml for western blotting

HC10- a mouse monoclonal antibody to unfolded MHC class I B and C alleles. Used at 10 μg/ml for immunoprecipitation and 1 μg/ml for western blotting (Stam et al., 1986).

Giles –a rabbit polyclonal antibody made to a N terminus peptide of human tapasin (a gift from B Gao, Institute of Molecular Medicine, Oxford). Used for Western blotting at a dilution of 1 in 500.

2.3. Peptides

The peptides used are described in Table 2.3. All peptides were synthesised by F-Moc using a Zinnser analytical synthesizer and provided as a lysophilised powder. Accompanying HPLC profiles showed them to be greater than 95% pure. Aliquots were made in 10% DMSO and stored at -20°C

2.4. Primers

Tapasin primers were ordered from Cruachem and made up to a concentration of 2μg/μl. The primers used for Conformation sensitive gel electrophoresis are

Allele	Peptide	Source
B*4402	SEIDTVAKY	Endogenous (DiBrino et al., 1995)
	SAIDTVAKY	As above
	SAIDTVAKA	As above
	AAASEIDTVAKY	As above
	AAIAAVAKY	As above
	ADIAAVAKY	As above
	AEIAAVAKY	As above
B*2705	GRAFVTIGK	HIV1 gp120 (Dedier et al., 2000)
	GRDFVTIGK	As above
	GRAFVTIGS	As above
	GRAFVTIGG	As above
	AAAGRAFVTIGK	As above
B*4405	SEGATPQDL	HIV1 P24 130-39 (Khanna et al., 1992)
	AEDKENYKKF	Hsp90α (Macdonald et al., 2003)
	FEDLRVLSF	Influenza A nucleoprotein (DiBrino et al., 1995)
	EENLLDFVRF	EBV EBNA3C 281-90 (Burrows et al., 1990)
	EEELLDVFRM	Endogenous (A .Purcell, unpublished data)

Table 2.3 – B*4402, B*2705 and B*4405 binding peptides

detailed in Table 2.4.1. The configuration of the primers used upon human tapasin is seen in Figure 2.4.1. The primers for subsequent polymorphic anaiyses were designed to have a single mismatch at the polymorphic base permitting Sequence Specific Priming (SSP) to be undertaken within a stringent PCR cycling design (Table 2.4.2). The configuration of the primers chosen for the subsequent exon4-intron4 haplotype analysis is seen in Figure 2.4.2.

The primate primers were as chosen at consensus sites between human and murine tapasin sequences (Table 2.4.3). It was assumed that such homology would also exist between human and monkey tapasin.

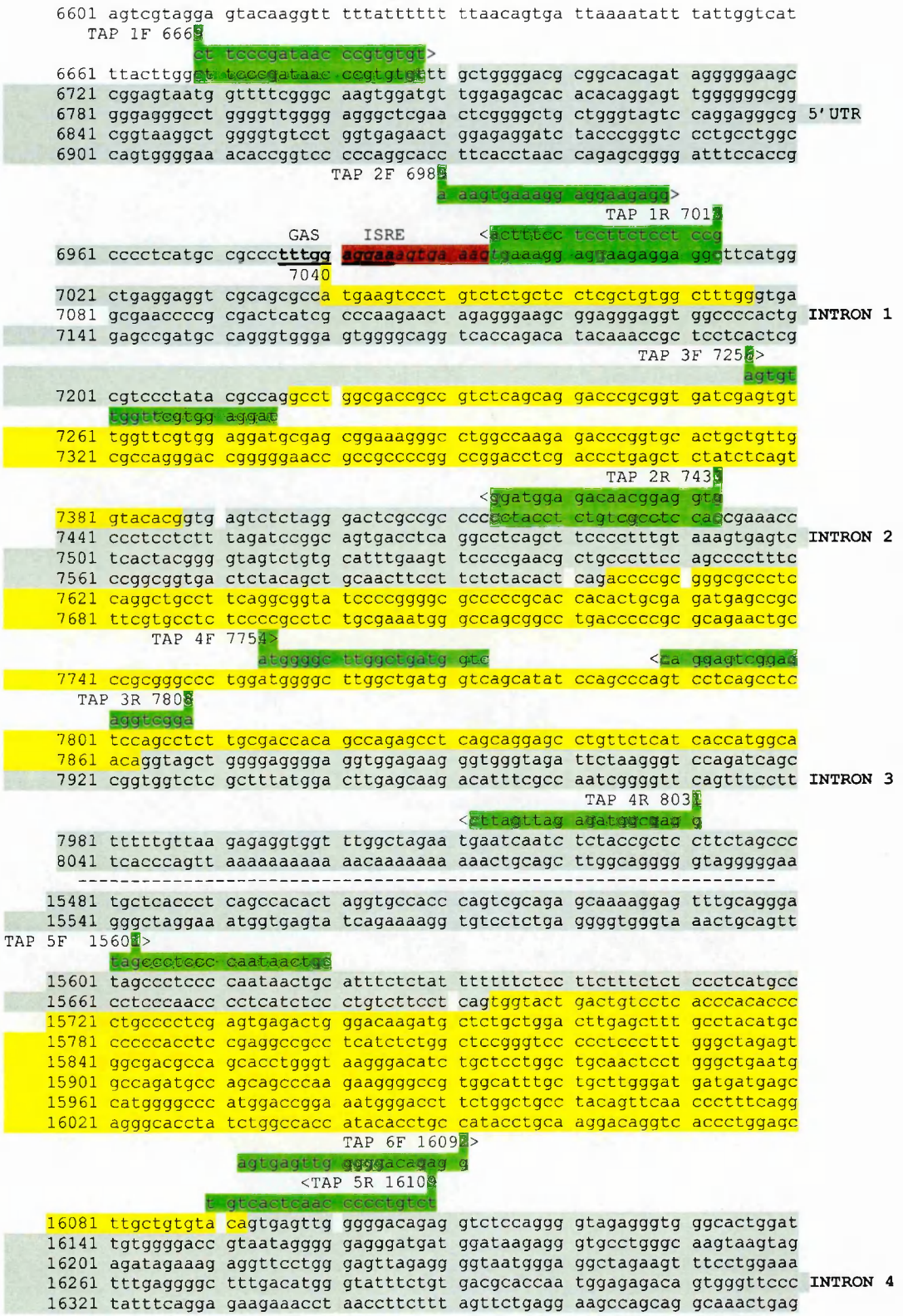
2.5. Vectors

Genomic B*4402 and B*2705 were contained within the puc13 vector (Weiss et al., 1985). Human tapasin cDNA was cloned into both pMCFR.puro and pMCFR.neo vectors (Ortmann et al., 1997). The tapasin contained the vector was the *01 (AGA) allele. The soluble tapasin vector was as previously described (Lehner et al., 1998). The ICP47 gene was cloned into the pMCFR.puro vector. B4402 was mutated to B*4405 using site directed mutagensis and cloned into the RSV.5neo vector.

Tapasin pair	Primer sequence	Amplified sequence	Tapasin region
TAP 1F	CTTCCCgATAACCCgTgTgT	6669 -7013	5' UT
TAP 1R	gCCTCCTCTTCCTCCTTTCA		
TAP 2F	AAAgTgAAAggAggAAgAgg	6989 -7413	Ex1, In1,Ex2
TAP 2R	gTggAggCaACAgAggTAgg		
TAP 3F	AgTgTTggTTCgTggAggAT	7256 -7808	In2, Ex3
TAP 3R	AggCTggAgAggCTgAggAC		
TAP 4F	ATggggCTTggCTgATggTC	7754 - 8031	Ex3, In3 (part)
TAP 4R	ggAgCggTAgAgATTgATTC		
TAP 5F	TAgCCCTCCCCAATAACTgC	15601-16109	Ex 4
TAP 5R	TCTgTCCCCCAACTCACTgT		
TAP 6F	AgTgAgTTgggggACAgAgg	16092 -16438	In4
TAP 6R	gggAAAgAggACgAAATgAg		
TAP 7F	gATTTCCCTATgCTCATTTC	16407 -16667	Ex5
TAP 7R	TgCCCAgAgAggCTgACAgA		
TAP 8F	CTggAggTggAgTgggAACT	16549 -17103	Ex5, In5, E6
TAP 8R	ggAgTAggTggggAAAgTCC		
TAP 9F	ggCTgggCTggTAAgTgT	16944 -17193	In6,Ex7,In7 (part)
TAP 9R	TgggTTTTgAgATAggAT		
TAP 10F	TCCTTCTCCCATCATCTgTg	19201 -19680	In7(part),Ex8, 3' UT
TAP 10R	CCCgTCTCTACTAAAAATAC		

Table 2.4.1 – Tapasin primers used for CSGE.

Human Tapasin
Accession Z97184



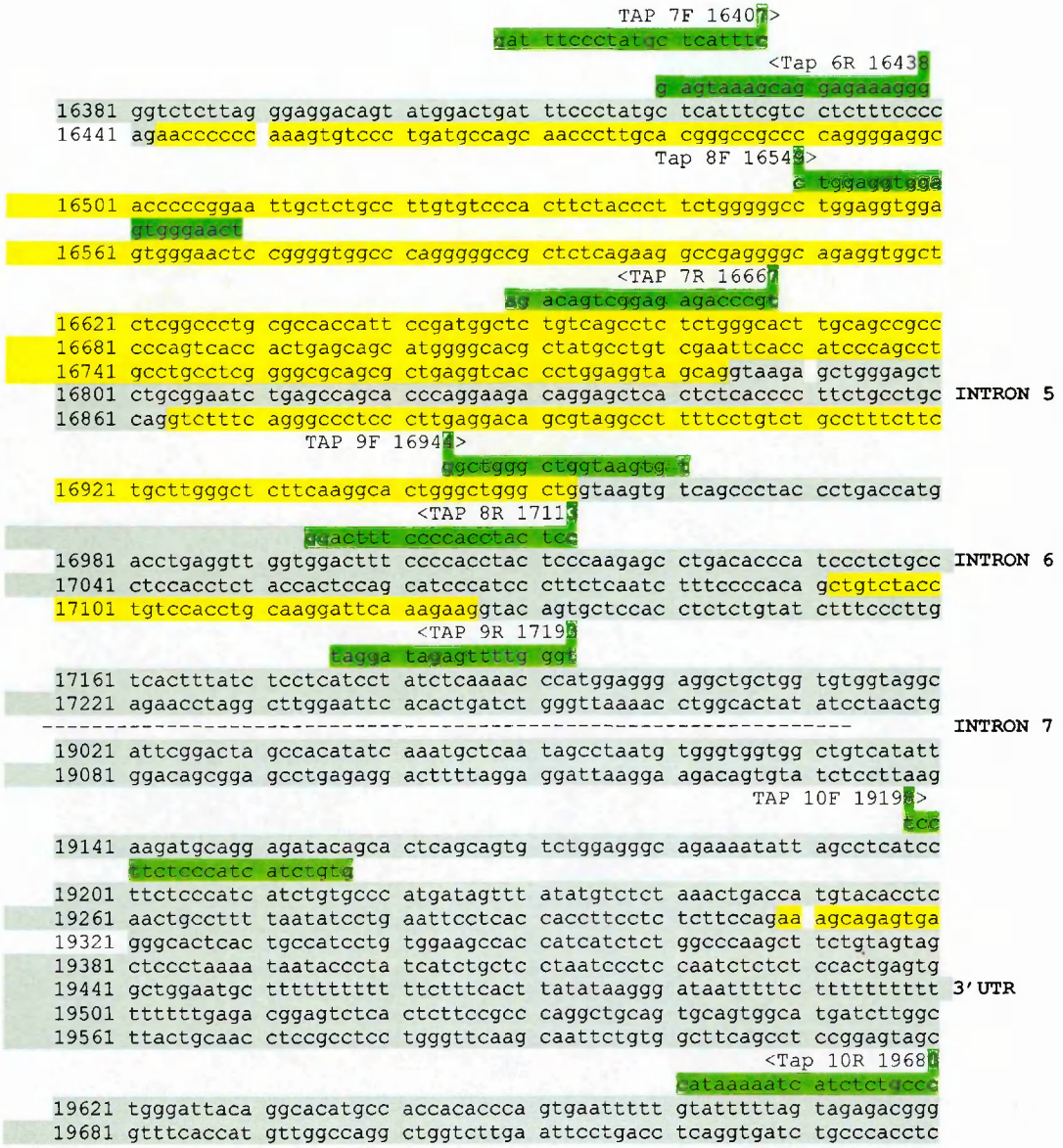


Figure 2.4.1 – Primer arrangement for CSGE analysis of Tapasin.

The primers were designed upon the human Tapasin sequence (Accession Number Z97184). The exon sequences are :Ex 1 7040 -7076, Ex 2 7217 -7387,Ex 3 7604 -7864,Ex 4 15694 - 16092,Ex 5 16443 -16784,Ex 6 16864 -16953,Ex 7 17092 -17126,Ex 8 19309 -19320. The intron sequences are colour coded grey, the exons yellow and the primers green.
The IFN-γ activated sites is marked in bold whilst the Interferon stimulated response element is in italic

Tapasin region	Primer Sequence
Tapasin *01 (16003-g/Ex4)	gACCTTCTggCTgCCTAg
Tapasin *02 (16003-C/Ex4)	gACCTTCTggCTgCCTAC
Tapasin In4/16146-g	CCCTCCCCCTATTACggTC
Tapasin In4/16232-g	AAACTTCTAgCCTCCCATTAC
Tapasin In4/16317/T	TCTTCTCCTgAAATAgggA
Tapasin In4/16146/T	CCCTCCCCCTATTACggTA
Tapasin In4/16232/A	gAAACTTCTAgCCTCCCATTAT
Tapasin In4/16317/A	TCTTCTCCTgAAATAgggT
Tapasin In6/17041/C	ACACCCATAAATATgCCC
Tapasin In6/17041/T	gACACCCATAAATATgCCT

Table 2.4.2 – Tapasin primers used for SSP.

Human Tapasin
Accession Z97184

Exon 4 –Intron 4 consensus	15986 (16003-C/G) -16309 = 323 bp

Exon 4 –Intron 4	15986 (16003-C/G) -16164 = 178 bp
Exon 4 –Intron 4	15986 (16003-C/G) -16252 = 266 bp
Exon 4 –Intron 4	15986 (16003-C/G) -16338 = 352 bp

Figure 2.4.2. – Configuration of the primers used for Tapasin exon 4-intron 4 haplotype analysis.

The primers used for the haplotypes analysis are seen above. The colour coding was as follows XXXXXXX – Intron, XXXXXXX – Exon, XXXXXXX - Primer similarity, XXXXXXX - Polymorphic base and XXXXXXX - Primer mismatch/identity for SSP.

Primate Tapasin primer pair	Primer Sequence	Amplified region	Tapasin Region
5'UTR	CACCTAACCAgAgCgggg	6933-7914	Ex1,In1,Ex2,In2,Ex3.
In3R	CTggACCCTTAgAATCTACCC		
In 3F	gCCCTCCCCAATAACTgC	15603-16658	Ex4,In4,Ex5 (part)
Ex 5R	ggCTgACAgAgCCATCgg		
Ex5F	gCCCCAggggAggCAC	16487-17144	Ex5,In5,Ex6,In6,Ex7
In 7R	AgAggTggAgCACTgT		
In 7F	CCTCACCACCTTCCTCTCTT	19285-19363	Ex8
3'UTR	gCCAgAgATgATggTggC		

Table 2.4.3 – Tapasin primers used for monkey Tapasin sequencing.

2.6. DNA preparation and Conformational Specific Gel Electrophoresis (CSGE)

The search for mutations in the tapasin gene was performed using conformational specific gel electrophoresis (CSGE). DNA was extracted from venous blood samples using a standard sucrose-lysis procedure. PCR primers were designed to amplify 10 regions of the tapasin gene between positions 6669-19680 (cosmid F0811 numbering) of tapasin (GenBank accession number Z97184) including the 5' and 3' untranslated regions, all 8 exons and introns 1,2,4,5 and 6. The large introns 3 and 7 were only partially amplified. CSGE was performed as previously described (Korkko et al., 1998). All samples with bandshifts were sequenced in duplicate and in forward and reverse orientations after re-amplification of the appropriate exon from genomic DNA in the PCR. PCR products were sequenced using the ABI Ready Reaction Dye Terminator Cycle Sequencing Kit and the ABI 377 Prism sequencer.

2.7. Mutational analysis of the tapasin gene

DNA extraction was carried out using a modified salting out procedure. MHC class I genotyping was performed using a polymerase chain reaction (PCR) sequence-specific method previously described for HLA typing (Bunce et al., 1995). Primers used were designed with appropriate mismatches to detect the 5

polymorphisms studied. The control primer used was specific for a constant region of HLA DRB1 (796 base product).

PCR was carried out in a final volume of 13 μL in 96-well plates under conditions identical to HLA typing. The final concentrations of the reaction components were as follows: 200μM of each dNTP, 2mM $MgCl_2$, 67mM Tris base, pH 8.8, 16.6 mM ammonium sulphate, 0.1% (vol/vol) Tween 20 between 0.01 and 0.1 μg DNA and 0.2 units of Taq polymerase (Advanced Biotechnology, London, UK). PCR amplifications were carried out in MJ Research 96 V machines under the following cycling conditions: 1 minute at 96°C; 5 cycles of 25 seconds at 96°C, 45 seconds at 70 °C, 45 seconds at 72 °C ; 21 cycles of 25 seconds at 96 °C , 50 seconds at 65 °C , and 45 seconds at 72 °C ; 4 cycles of 25 seconds at 96 °C , 60 seconds at 55 °C , and 120 seconds at 72 °C . An aliquot of the PCR reaction plus 6μL of loading dye was loaded into a 1% agarose gel (containing ethidium bromide) in 0.5 -Borate EDTA solution. Gels were electrophoresed for 30 minutes at 200 V/cm^2. Gels were photographed under ultraviolet light (320nm). Each gel was scored for the presence or absence of an allele specific band, provided that the control amplicon was present.

2.8. Polymorphic analysis of the tapasin gene

The analysis of a linkage between the exon4 and intron 4 polymorphisms was undertaken with each sequence specific primer. The configuration of the chosen

primers is seen in Figure 3.1.2. The PCR conditions were identical to those detailed in 2.7.

2.9. Polymorphic analysis and sequence analysis of primate tapasin

The primers for primate tapasin were designed using areas of maximal human/mouse homology and assuming that such homology would be present in the evolutionary closer macaque sequences. All primers were chosen to anneal at 60°C and the reactions were undertaken in 50μl eppendorf tubes. The reactions included 5 μl NH_4 reaction buffer (Bioline), 2 μl $MgCl_2$ 50mM (Bioline), 2 μl 25mM dNTp mix (Bioline), 38 μl H_2O 1 μmole/ μl of forward and reverse primers, 0.5 μl of Taq polymerase (Bioline) and 0.5 μl of template DNA. The PCR amplifications were carried out in an Eppendorf gradient mastercycler under the following conditions: 35 cycles of 60 seconds at 94°C; 60 seconds at 58°C and 60 seconds at 68 °C. PCR products were purified according to the manufacturer's instructions (QUIagen QIAquick PCR purification kit. All sequencing was undertaken through the Oxford University Biochemistry Department sequencing facility. The sequence analysis was undertaken using the internet sequence alignment program CLUSTALW.

2.10. Statistical methods

Analysis of the relationship between tapasin and MHC class I genotypes was made using the Chi-squared test implemented in the Knowledge Seeker program (Knowledge Seeker IV version 4.2.1, Angloss International Limited). All allele frequencies were calculated by direct counting. A $\chi 2$ value of greater than 6.63 ($P<0.01$:DF =1) was considered as a significant association.

2.11. Cell transfections

721.220 cell lines co-expressing B*4402 or B*2705 were transfected by electroporation at 220V and 960μF with the soluble tapasin and ICP47 vectors. The cell lines were selected with 0.5mg/ml of G418 or 4 μg/ml of puromycin. All experiments were undertaken on bulk populations that were selected using magnetic beads (Dynabead) that had been coated with the Bw4 antibody (116.5.28).

2.12. Thermostability assays

For thermostability assays, the relevant cell transfectants were starved in methionine and cysteine-free medium for 40min and pulsed with [^{35}S]-methionine/cysteine (Promix®, Amersham, U.K.) at 100 μCi/ml/10^7cells for 30min. Cells were collected, washed in cold PBS and lysed in Triton X-100 buffer

(1% Triton X-100, 10mMTris[pH7.4], 150 mM NaCl with PMSF) for 30min at 4°C. To test for stabilisation of MHC class I complexes by variant peptides, this incubation step was carried out in the presence of 50μM of the relevant peptide. After cell nuclei had been centrifuged, cell lysates were precleared with protein A-sepharose beads (Sigma, St. Louis, MO, St. Louis) for 30min. Following this, equal aliquots of cell extracts (derived from $2x10^6$ cells) were loaded into thin walled eppendorfs and kept at either 4°C or placed across a temperature gradient in a thermocycler (Mastercycler Gradient, Eppendorf). The samples were heated at required temperatures for 12 minutes and then returned immediately to 4°C. Thereafter, mAb W6/32 and protein A-sepharose beads were added for 1 hour at 4°C with gentle rotation. The beads were washed once in 450mM NaCl solution containing 0.1% Triton X-100, 10mM Tris buffer (pH7.4) and once in 10mM Tris Buffer (pH7.4). The beads were heated at 80°C in 20μl of x2 LDS sample buffer (pH8.5) for 5min and proteins were subsequently separated by 10% non-reduced SDS-PAGE gel. The gel was then fixed, soaked in Amplify (Amersham, U.K.) and dried. The images were analysed with a Fujifilm FLA-2000 phosphorimager. The arbitrary intensity units (AIU) of each heavy chain signal was quantified and the relative stability of class I complexes after heating is expressed as the AIU after heating in relation to the relevant AIU at 4°C. For pulse-chase thermostability assays, cells were labelled for 5min with [^{35}S]-methionine/cysteine at 100 μCi/ml/10^7 cells and then chased in excess methionine (2mM) and cysteine (2mM). At indicated time points, cells were harvested and lysed in Triton X-100 buffer. Equal aliquots of cell extracts derived

from $2x10^6$ cells were kept at either 4°C or heated at indicated temperatures for 12min. Thereafter, samples were treated as above. Where cell extracts had been heated to 50°C, the samples were spun at 21,000g for 10 min to remove any heat-induced precipitates prior to immunoprecipitation.

2.13. Pulse chase radiolabelling

Cells were starved in methionine and cysteine-free medium for 40min and pulsed with [^{35}S]-methionine/cysteine (Promix®, Amersham, U.K.) at 100 μCi/ml/10^7cells for 5 minutes. The cells were then chased for 15, 30, 60 and 120 minutes in RPMI 1640 media supplemented with 10% FCS and 2mM methionine/cysteine. For short pulse chase experiments (6,12 and 18 minutes chase) a 2 minute pulse label was used. At each time point, cell aliquots were removed, washed in ice cold PBS and lysed in 1% Triton X-100 buffer (pH 7.4). Postnuclear supernatants were precleared for 1 hour at 4°C prior to immunoprecipitation with W6/32 conjugated Sepharose beads for 1 hour at 4°C. Washed precipitates were resuspended in 20uL of 50mM sodium citrate buffer (pH 5.5), containing 0.2% SDS, heated to 80°C for 5 minutes, and digested with 5mU endoglycosidase H (Roche) overnight at 37°C. Samples were analysed on a 10% SDS-PAGE gel. The gel was then fixed, soaked in Amplify (Amersham, U.K.) and dried. The images were analysed with a Fujifilm FLA-2000 phosphorimager. The data was then plotted as the % of maximal endoglycosidase H resistant material seen at each time point.

2.14. Cell surface iodiniation

Cells ($1x10^6$) were washed once in Dulbecco's PBS (Sigma, St. Louis, MO) and radiolabelled with 0.5mCi 125-Iodine in 1ml of Dulbecco's PBS within an Iodo-Gen pre-coated tube (Pierce) for 15min at room temperature with gentle agitation. Cells were then washed twice in Dulbecco's PBS containing 5mM KI, lysed in Triton X-100/TBS buffer and subjected to immunoprecipitation as described above.

2.15 Flow Cytometry

Cells were stained with mAb 116.5.28 for HLA-B4402 followed by goat anti–mouse antiserum conjugated to FITC (Sigma, St. Louis, MO). $2x10^4$ cells were analysed for each histogram using a FACS analyser (Becton Dickinson, CA). For cell surface decay experiments, $5x10^6$ cells were treated with 10μg/ml BFA and cultured at 37°C. Equal aliquots of cells were withdrawn at 0, 8, 16 and 24hrs, stained with mAb and fixed with 1% paraformaldehyde in PBS. Cell surface

decay of class I complexes was expressed as mean channel fluorescence (MCF) at these time points in relation to the MCF at time 0.

2.14 Western blotting

Cells were grown to a density of $3x10^5$/ml prior to harvesting for immunoblotting. The cells were spun at 1300rpm for 3 mins at 4°C. The cells were washed in PBS and lysed in ice cold Tris lysis buffer (pH 7.5) (150m NaCl,5mM EDTA, 20mM TrisHCl, 2mM PMSF and 5mM iodoacetamide) containing 1% triton X. The cells were routinely lysed at a concentration of 10^7/ml. Following 30 minutes at 4°C the samples were centrifuged at 13000rpm (4°C) for 10 minutes. Samples were then mixed with an equal volume of x2 sample buffer (10% SDS, 5% β-mercaptoethanol (2-ME), 50mM Tris (pH6.8), 30% glycerol and 0.005% bromophenol blue). Following heating for 5 minutes at 95°C the samples were loaded onto a 10% SDS-polyacrrylamide gel. A stacking gel (0.125M Tris pH 6.8 ,13% Bisacrylamide (30%),0.1% SDS) and 10% resolving gel (0.375M pH8.6, 30% bisacrylamie (30%), 0.1% SDS) were polymerised with 10% ammonium persulphate and TEMED and made fresh for each gel. The gels were run at 200V for 1 hour within a Novex cassette apparatus. Transfer was undertaken for 90 mins at 26V to nitrocellulose membranes (Hybond C, Amersham). Following transfer the gel was stained with Ponceau red. The membrane was then blocked

in 5% non fat milk in 0.05% PBS tween (blocking buffer) for 1 hour at 4°C. The membrane was washed 3 times in 20 ml of 0.05% PBS tween. Primary antibody was diluted to the appropriate concentration in blocking buffer and the membrane was soaked in this for 90 minutes. The washing procedure was then repeated. The secondary species and isotype specifc antibody was then diluted appropriately in the blocking buffer and added to the membrane for 1 hour. Following 3 further washes the membrane was soaked in a chemiluminescent detection reagent (ECL, Amersham) and exposed to a CCD camera for image capture (Fuji BAS FLA2000)

Chapter 3 RESULTS

3.1 INVESTIGATION OF TAPASIN POLYMORPHISMS

3.1.1 DETERMINATION OF TAPASIN POLYMORPHIC FORMS

The investigation of polymorphic variants of tapasin was undertaken by conformational sensitive gel electrophoresis in collaboration with Dr R Houlston and S Bevan at the Institute of Cancer Research, Sutton. Genomic DNA from 40 healthy Caucasian U.K. individuals aged less than 55 years of aged was used to screen for sequence variation in the tapasin gene. Ten primer pairs were designed to amplify all 8 tapasin exons in addition to the 5'UTR, 3'UTR regions and introns 1,2,4,5 and 6. Conformational sensitive gel electrophoresis was undertaken to screen for polymorphisms (Korkko et al., 1998). This technique depends upon the formation of a mismatched dsDNA which will run differently to that of a perfectly matched dsDNA molecule through an acrylamide gel. Following this approach 43 CSGE band shifts were identified within the 40 samples (Table 3.1.1). Most of these were located within exon 4 and intron 4. All samples with a band shift were sequenced in forward and reverse orientations following re-amplification of the appropriate PCR product to confirm a true positive result by CSGE.

Primer pair	Sample Number (Total numbers in brackets)	Confirmed by sequencing
TAP 1	None	None
TAP 2	1 (1)	None
TAP 3	1 (1)	None
TAP 4	None	
TAP 5	3,5,6,7,8,9,13,16,17,18, 20,21,23,24,29,30,32,39 (18)	Confirmed by sequencing
TAP 6	1,3,5,6,7,9,10,11,13,17, 21,22,25,28,29,31,34,37,39 (19)	Confirmed by sequencing
TAP 7	None	
TAP 8	18,19,34 (3)	None confirmed
TAP 9	16 (1)	Confirmed by sequencing
TAP 10	None	
TOTAL	43	

Table 3.1.1 – Potential Tapasin polymorphisms identified by conformational sensitive gel electrophoresis (CSGE).

A total of 43 CSGE band shifts were identified in a sample of 40 DNA samples. The majority of the bandshifts were seen with primers that targeted exon 4 and intron 4 of the tapasin gene

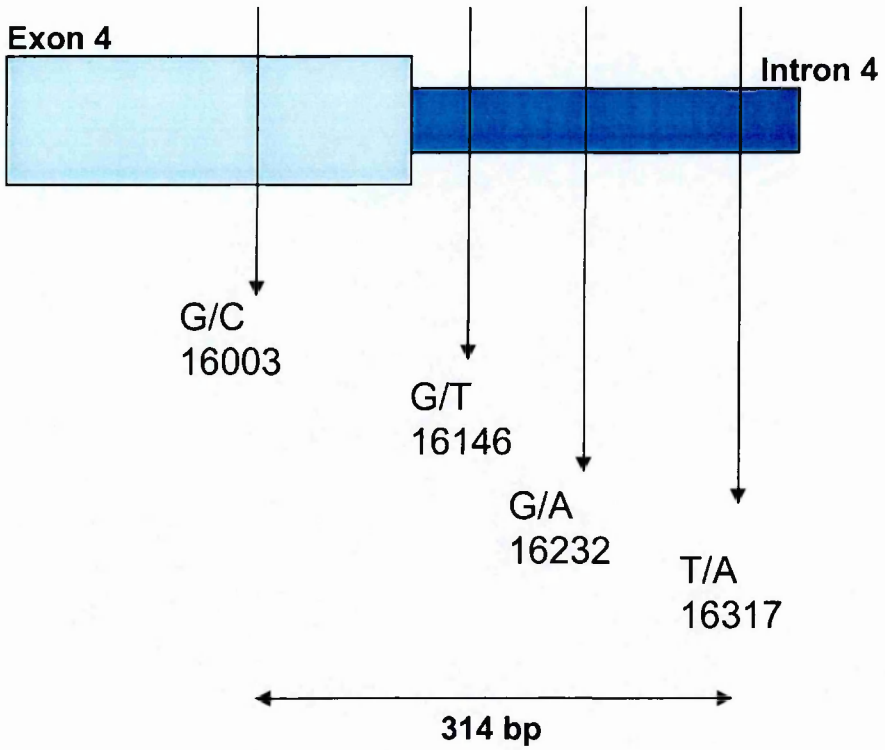
Direct sequencing did not identify any polymorphisms to account for the bandshifts seen with the TAP 2, 3 and 8 primer pairs. It is well recognised that the higher sensitivity of CSGE is offset by a higher false positive rate. The bandshifts identified with the TAP 5 pair were all secondary to a Cytosine (C) to Guanine (G) change at position 16003 within exon 4 (Accession number Z97183). This represented a non-conservative codon change of a Threonine (ACA) to arginine (AGA) at amino acid position 240. The 16003-G allele was subsequently termed Tapasin *01 and the 16003-C, Tapasin *02. The TAP 6 bandshifts were secondary to one of 3 polymorphisms identified within intron 4. These were a Guanine to Thymidine (T) change at 16146, Guanine to Adenosine (A) change at 16232 and Thymidine to Adenosine change at 16317 The TAP 9 bandshift was confirmed as a C>T change at position 17041.

Sequence specific primers were then designed to specifically anneal to the identified polymorphic variants. The original 40 samples were again analysed to confirm the specificity of the primers and to determine whether any co-association of polymorphisms existed. The primers were multiplexed to look for a linkage between the exonic dimorphism and the three intronic dimorphisms. This would allow for the determination of an exonic-intronic 4 haplotype for each allele. This configuration is seen in Figure 3.1. Of the original 40 samples used for CSGE, 25 were available for typing in this manner. The frequency of the dimorphism was 8/25 (0.32) for the 16003-G homozygote, 4/25 (0.16) for the 16003-C homozygote and 13/25(0.52) as heterozygotes. It became apparent

that of the many intron 4 sequences that were mathematically feasible, only 4 sequences were identifiable (Figure 3.1.1). The G/G homozygotes samples included 3 TAA, 4 GGT and a single TGA intronic sequence. Within the 4 C/C homozygotes all had the GGT wild type intronic sequence. This small initial sample suggested that the Tapasin*02 variant may be linked to a fixed intronic 4 sequence. This initial cohort analysis was investigated further through a review of 81 DNA samples obtained from cadaveric renal transplant donors.

3.1.2 ANALYSIS OF THE FREQUENCY OF TAPASIN POLYMORPHIC VARIANTS AND THE EXON 4-INTRON 4 LINKAGE.

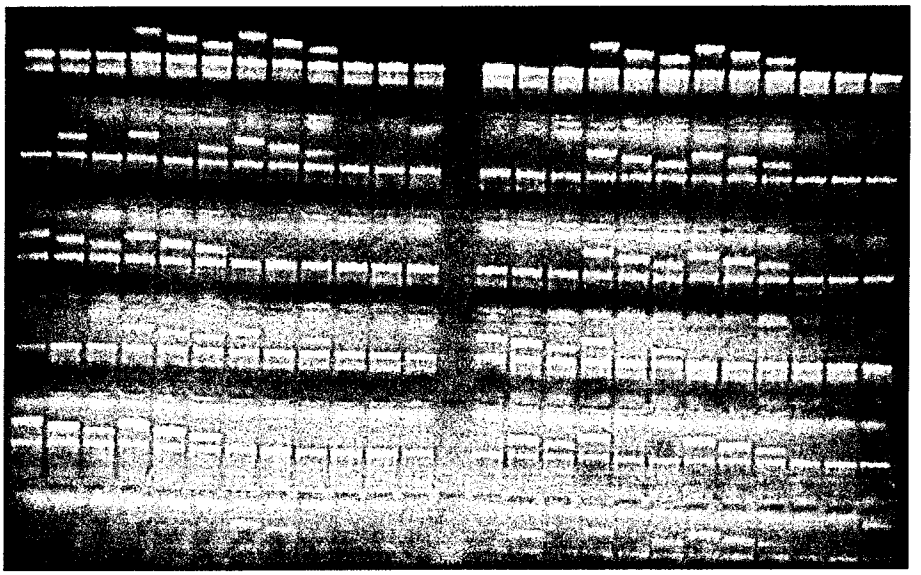
The frequency of the 5 polymorphisms was determined in a series of 81 genomic DNA samples. The PCR typing was set up to determine the tapasin allele present and its linkage to the three intronic 4 dimorphisms. The schematic layout of the PCR approach and a representative PCR experimental result is seen in Figure 3.1.2. All 81 samples were successfully amplified and the results are seen in Table 3.1.2a. The Tapasin*01 allele had a frequency of 0.47 and the *02 allele of 0.53. The genotype frequency was for *01*01 = 0.22, *01*02 = 0.49 and *02*02= 0.28. The four intron haplotypes were identified within this population at a frequency of GGT = 0.54, TAA = 0.23, TGA = 0.22 and TGT = 0.006. Once again all the *02 alleles were associated with the wild type GGT intron 4 sequence. Only one of the *01 alleles was associated with this sequence. The



Intron 4	16146	16232	16317
1	G	G	T
2	T	A	A
3	T	G	A
4	T	G	T

Figure 3.1.1 - Exon 4-Intron 4 haplotype configuration

The exon 4 dimorphism and intron 4 dimorphisms extended over less than 350bp of DNA. Of the many permutations of intronic sequence that could possibly be identified only 4 were seen.



Forward Primer 16003- G EXON 4	Forward Primer 16003- C EXON 4	Forward Primer 16003- G EXON 4	Forward Primer 16003-C EXON 4

Reverse Primer INTRON 4

G	G	T	T	A	A	G	G	T	T	A	A		G	G	T	T	A	A	G	G	T	T	A	A

G-TAA C-GGT	G-TAA C-GGT
G-TGA C-GGT	G-TAA C-GGT
G-GGT G-TAA	G-TAA C-GGT
G-TAA C-G*T	G-GGT G-TGA
G-GGT G-TAA	G-TGT C-GGT
REPEAT	G-TGA C-GGT

Figure 3.1.2–Representative multiplex PCR gel of Tapasin exon 4 and intron 4 polymorphisms

Each DNA sample is tested with an exon 4 forward primer and each of the intronic primers which are specific for each dimorphism. An exon-intron 4 haplotype can then ascribed to each sample.

most frequent intronic haplotypes associated with the *01 allele were TAA =0.5 and TGA =0.47

There are only 314 bp between the exon 4 dimorphism and the last of the three intronic polymorphisms (Figure 3.1.1). The singular linkage between the *02 allele and the intronic haplotypes GGT is in contrast to the *01 alleles which is seen with both the TAA and TGA intronic sequence. A second set of DNA samples were then chosen, to assess whether this linkage could be repeatedly seen. This second donor set (from the Oxford transplant centre DNA bank of cadaveric renal donors) had been previously typed across the MHC region for MHC class I and II loci. A similar linkage was seen between the exon 4 dimorphism and intron 4 sequences within this control set. This data is shown in Table 3.1.2b

An analysis was then undertaken to look for a linkage between the tapasin polymorphisms and the MHC class I and II genes. The Chi squared (X^2)statistical analysis was used to determine whether the frequencies of the polymorphisms associated with a particular allele differed significantly from the frequencies calculated from the control group

3.2 DETERMINATION OF TAPASIN LINKAGE WITH MHC CLASS I AND II ALLELES

CONTROLS I		
POLYMORPHISM	**SAMPLE NUMBER =81**	**FREQUENCY**
16003-G	76/162	0.47
16003-C	86/162	0.53
GG	18/81	0.22
CC	23/81	0.28
CG	40/81	0.49
GGT	87/162	0.54
TAA	38/162	0.23
TGA	36/162	0.22
TGT	1/162	0.006
C-GGT	86/86	1.0
G-TAA	38/76	0.5
G-TGA	36/76	0.47
G-TGT	1/76	0.013
G-GGT	1/76	0.013

Table 3.1.2a - Frequency of the Tapasin dimorphism and intron 4 linkage in normal controls.

The frequency of the tapasin dimorphism and intron 4 dimorphisms are seen. The tapasin dimorphisms segregate in accordance with the Hardy-Weinburg equation whereas the intronic mutations appear to be in linkage with particular tapasin exon 4 variants.

Integrated Control results						
Polymorphism	**Controls I**		**Control II**		**Combined I & II**	
16003-G	76/162	**0.47**	100/164	**0.61**	176/326	***0.54***
16003-C	86/162	**0.53**	64/164	**0.39**	150/326	***0.46***
GG	18/81	**0.22**	32/82	**0.39**	50/163	***0.31***
CC	23/81	**0.28**	14/82	**0.17**	37/163	***0.23***
CG	40/81	**0.49**	36/82	**0.44**	76/163	***0.47***
C-GGT	86/86	1.0	63/63	1.0	149/149	1.0
G-TAA	38/76	0.5	45/99	0.45	83/175	0.47
G-TGA	36/76	0.47	50/99	0.51	86/175	0.49
G-TGT	1/76	0.013	0/99	0.00	1/175	0.006
G-GGT	1/76	0.013	4/99	0.04	5/175	0.028
G-TAT	0/76	0.0	0/99	0.00	0/175	0.0

Table 3.1.2b - Integrated control results for the Tapasin exon 4/ intron 4 dimorphisms and linkage

Certain MHC class I alleles form extended haplotypes where they are found together at a higher frequency than would be predicted by their individual allele frequencies (Price et al., 1999). Typical MHC linkage disequilibria include the A1B8DR3 and A3B7DR15 associations. It was of note that the former haplotypes contained tapasin dependent alleles whilst the latter tapasin independent alleles (Greenwood et al., 1994). We undertook a Chi squared analysis of the frequency of the tapasin polymorphisms with the single alleles and the extended haplotypes. However, no single MHC class I allele or haplotype was significantly associated ($P<0.01$) with any of the tapasin polymorphisms (Table 3.2.1).

However, on consideration of the MHC class II alleles some significant associations were seen (Table 3.2.2). Of the control samples that were available for MHC class II typing, 2 interesting associations were seen. Firstly a positive association was seen between DQ6 and Tapasin *02. Of the 30 DQ6 positive samples only 1 was identified within the Tapasin *02 allele. The negative association of the DQ6 (DQB1*0601-0615) with the G Tapasin allele suggests that the DQ6 may exist in cis with the Tapasin *02 allele. DP*0101 also appeared to be associated with the Tapasin *02 allele. Of the 7 DP*0101 samples, 5 were seen within the CC homozygotes. The Tapasin*01 allele showed weaker linkages to the DR4 and DP*0402 alleles.

MHC Class I Association					
	Control Group	**16003-C**	**16003-G**	**G-TAA**	**G-TGA**
Control Group	**81 (100%)**	**63 (78%)**	**58 (72%)**	**31 (38%)**	**34 (42%)**
A1	21 (26%)	15 (71%)	14 (66%)	10 (48%)	6 (29%)
A3	22 (27%)	7 (77%)	18 (82%)	11 (50%)	9 (41%)
B7	24 (30%)	22 (92%)	19 (79%)	7 (29%)	12 (50%)
B8	16 (20%)	13 (81%)	10 (63%)	7 (44%)	4 (25%)
B27	6 (7%)	4 (67%)	4 (67%)	1 (17%)	4 (67%)
B4402	10 (12%)	5 (50%)	8 (80%)	7 (70%)	3 (30%)
Cw701	19 (235)	16 (84%)	11 (58%)	7 (37%)	5 (26%)
Cw702	26 (22%)	24 (92%)	21 (81%)	8 (31%)	13 (50%)
A1B8Cw701	13 (16%)	10 (77%)	8 (62%)	6 (46%)	3 (23%)
A1B8DR3	9 (9%)	6 (66%)	5 (56%)	5 (56%)	1 (11%)
A3B7Cw702	11 (14%)	9 (82%)	10 (91%)	6 (55%)	4 (36%)
A3B7DR15	5 (6%)	5 (100%)	4 (80%)	1 (20%)	2 (40%)

Table 3.2.1 – Association of selected MHC class I alleles with tapasin polymorphisms

The Chi squared analysis showed no statistical association between any MHC class I allele or extended haplotype with either of the tapasin polymorphisms.

(i)

MHC class II Associations			
DQ6	**GG**	**Not GG**	**Total**
+ve	1	29	30
-ve	19	44	63
Total	20	73	93
CHI =8.40 Df=1 P=0.004			

MHC class II Associations			
DP*0101	**CC**	**Not CC**	**Total**
+ve	5	2	7
-ve	18	65	83
Total	23	67	90
CHI =8.4 Df=1 P=0.003			

(ii)

MHC class II Associations			
DR4	**GG**	**Not GG**	**Total**
+ve	11	16	27
-ve	7	56	63
Total	18	72	90
CHI =9.9 Df=1 P=0.001			

MHC class II Associations			
DP*0402	**CC**	**Not CC**	**Total**
+ve	1	12	13
-ve	17	29	46
Total	18	41	59
CHI =4.8 Df=1 P=0.03			

Table 3.2.2 – Association of selected MHC class II alleles with tapasin polymorphisms

(i) The Tapasin *02 allele appeared to be linkage with the DQ6 alleles. The DP allele, DP*0101, was also associated with Tapasin *02.

(ii) Tapasin*01 was associated with DR4 and DP*0402.

Following this initial analysis I undertook an analysis of 2 disease groups. The first group were individuals with autoimmune thyroid disease (AITD) and the second group, those with psoriasis. These groups were originally chosen secondary to the A1B8DR3 association with AITD and the Cw6 association with psoriasis. 170 individuals with AITD and 97 individuals with psoriasis were analysed. Once again all tapasin *02 alleles were associated with the GGT intronic haplotypes and the TGA and TAA intronic haplotypes were the most common within these disease groups. Chi squared testing showed no statistical association between the tapasin alleles and/or genotypes of these disease compared to those of the controls (data not shown).

3.3 INVESTIGATION OF PRIMATE TAPASIN POLYMORPHISMS

The conservation of the exon 4-intronic 4 haplotype across more 150 samples prompted its analysis in primates. Rhesus macaque monkey genomic DNA was obtained (through Dr Tom Hanke, Institute of Molecular Medicine, Oxford) and the tapasin dimorphism investigated. The original exon 4 forward primers were chosen as there was human to mouse homology across this region and a consensus exon 5 primer designed at a site of homology between mouse and human. It was assumed that such conservation would be maintained across the human-primate sequences. 67 samples were analysed and all were homozygous for the tapasin AGA (arginine –human tapasin*01) codon. To ascertain which intron 4 sequences were associated with this codon, 4 genomic

DNA's where sequenced across this region. These all showed the presence of the GGT sequence at positions 16146, 16232 and 16147 respectively. It appears that in primate tapasin the exon 4 intron 4 haplotype is of the form G-GGT which is the most uncommon haplotypes within the human haplotypes identified.

To further analyse these differences between monkey and human tapasin I undertook a sequencing project on primate tapasin. Due to the availability of only genomic primate DNA, the sequencing consisted of aligning the mouse and human introns/exons and designing primers that were directed at conserved regions based on the assumption that such regions would also be conserved in the primate DNA. This sequencing approach allowed for the determination of all the primate tapasin gene apart from that responsible for the first 10 residues of exon 4. Despite multiple attempts with different intron 3 primers, this sequence could not be successfully amplified and sequenced. A final sequence comparison shows a 96% homology between human and primate tapasin (Figure 3.3). Of the 15 amino acid differences between the 2 proteins, most occur within exons 2 and 3. Within exon 4 there is a single amino acid change (Proline to Alanine) and a threonine seen at the human dimorphic residue. The transmembrane and cytosolic regions are almost identical apart from a conservative isoleucine to valine change in the exon 6 encoded region. In comparison to mouse tapasin where there are over 30 amino acid differences compared to human tapasin, monkey tapasin provides a better platform to

(i)

```
                                        +1
H - MKSLSLLLAVALGLATAVSAGPAVIECWFVEDASGKGLAKRPGALLLRQG
M -MKSLSLLLAVALGLATAVSAGPAVIECWFVEDTSGKGLAKRPGALLLRQG
                                      Ex3
H - PGEPPPRPDLDPELYLSVHDPAGALQAAFRRYPRGAPAPHCEMSRFVPLP
M -QGEPPPRPDLDPELYLNVHDPAGFLQAAFRRYPRDAPAPHCEMSRFVPLP

H - ASAKWASGLTPAQNCPRALDGAWLMVSISSPVLSLSSLLRPQPEPQQEPV
M -ASANWASGLTPARNCPRALDGAWLMVSMSSPVLSLSSLLRRQPEPQQEPV
            Ex4
H -LITMATVVLTVLTHTPAPRVRLGQDALLDLSFAYMPPTSEAASSLAPGPP
M -LITMAT----------APRVRLGQDALLDLSFAYMPPTSEAASSLAAGPP

H - PFGLEWRRQHLGKGHLLLAATPGLNGQMPAAQEGAVAFAAWDDDEPWGPW
M - PFGLEWRRQHLGKGHLLLAATPGLNGQMPAAQEGAVAFAAWDDDEPWGPW
                    *                                  Ex5
H - TGNGTFWLPTVQPFQEGTYLATIHLPYLQGQVTLELAVYKPPKVSLMPAT
M -TGNGTFWLPTVQPFQEGTYLATIHLPYLQGQVTLELAVYKPPKVSLMPAT

H - LARAAPGEAPPELLCLVSHFYPSGGLEVEWELRGGPGGRSQKAEGQRWLS
M -LAWAAPGEAPPELLCLVSHFYPPGGLEVEWELRGGPGGRSQKAEGQRWLS

H - ALRHHSDGSVSLSGHLQPPPVTTEQHGARYACRIHHPSLPASGRSAEVTL
M -ALRHHSDGSVSLSGHLQPPPVPTEQHGARYACRIHHPSLPASGRSAEVTL
        Ex6                                Ex7              Ex8(+428)
H - EVAGLSGPSLEDSVGLFLSAFLLLGLFKALGWAAVYLSTCKDSKKKAE
M - EVAGLSGPSLEDSIGLFLSAFLLLGLFKALGWAAVYLSTCKDSKKKAE
```

(ii)

Exon	1	2	3	4	5	6	7	8
Amino Acids	12	57	87	133	114	30	12	3
Coding changes	0	3	6	1	3	1	0	0

Figure 3.3. – Comparison of human and monkey Tapasin protein sequences.

There are 15 differences between human and monkey tapasin and these are highlighted in colour (* denotes human polymorphisms). (ii) Most of the polymorphisms are located in exons 2 and 3 where approximately 6% of the amino acids are different between the 2 species.

investigate key regions of the tapasin molecule that may be important for it's functions.

Following these observations I focussed on the development of a tapasin functional assay that could define the ability of tapasin to improve the peptide repertoire of MHC class I complexes.

CHAPTER 4

4.1 A thermostability assay for the assessment of Tapasin function

The nature of the MHC class I peptide cargo has previously been determined through peptide elution experiments and mass spectrometry of the peptide species (Engelhard et al., 2002; Rammensee et al., 1993). These biochemical process performed on lysed cells examined the cell surface and intracellular peptide species that survived the MHC class I complex purification procedures. Other inferences regarding the peptide cargo had been made through peptide addition experiments ('assembly assay') where exogenous peptide had been added to lysed cells and its ability to stabilise poorly peptide loaded complexes analysed by immunoprecipitation experiments (Townsend et al., 1990). Finally the effect of temperature on complex stability had been probed. This was initially reported through studies of mouse class I alleles from peptide transporter incompetent cell lines whose cell surface class I complexes increased upon cell culture at 26°C (Schumacher et al., 1990). This was followed by studies upon purified empty and peptide filled complexes analysed by circular dichroism spectroscopy (Fahnestock et al., 1992). Lastly intracellular complexes from pulse labelled cells were assessed for peptide occupancy by heating followed by immunoprecipitation with a conformation sensitive antibody (Neefjes et al.,

1993a; Tan et al., 1997). MHC class I complexes that carried peptides of optimal length and anchor residues were thermostable whilst those with suboptimal peptide or without peptide were thermolabile and were not recognised by the conformation sensitive antibody after incubation at 37°C. This latter methodology had been used to probe the early events in HC, β_2m and peptide binding and provided the opportunity of working with cells whose antigen processing machinery, namely tapasin, could be manipulated. I decided to develop this assay to probe the repertoire of peptides occupied by B*4402, B*2705 and B*4405 in the presence and absence of tapasin.

The initial experiment on the cell lines 220, 220.B*4402, 220. B*4402.Tapasin, 220.B*4402.soluble Tapasin and 220.B*4402.Tapasin.ICP47 is shown in Figure 4.1.1. The conformational sensitive antibody W6/32 was used to detect HC:β_2m complexes. Comparison of lanes 1 and 3 shows detection of both the transfected B*4402 allele and endogenous C*0102 allele. However the majority of the heavy chain detected is that of B*4402. To quantify this proportion an IEF gel was run to enable separation of the MHC class I heavy chains. This showed that at least 80% of the radiolabelled HC signal detected with the W6/32 immunoprecipitation was the transfected HC of the B allele within the 220 cell line series (Figure 4.1.2). The quantitation of the HC bands in Figure 4.1.1 showed a spectrum of MHC class I stability. The B*4402 complexes were very stable at 37^{0}C when loaded in the presence of tapasin as compared to the absence of tapasin (95% stability versus 12% stability). Of additional interest

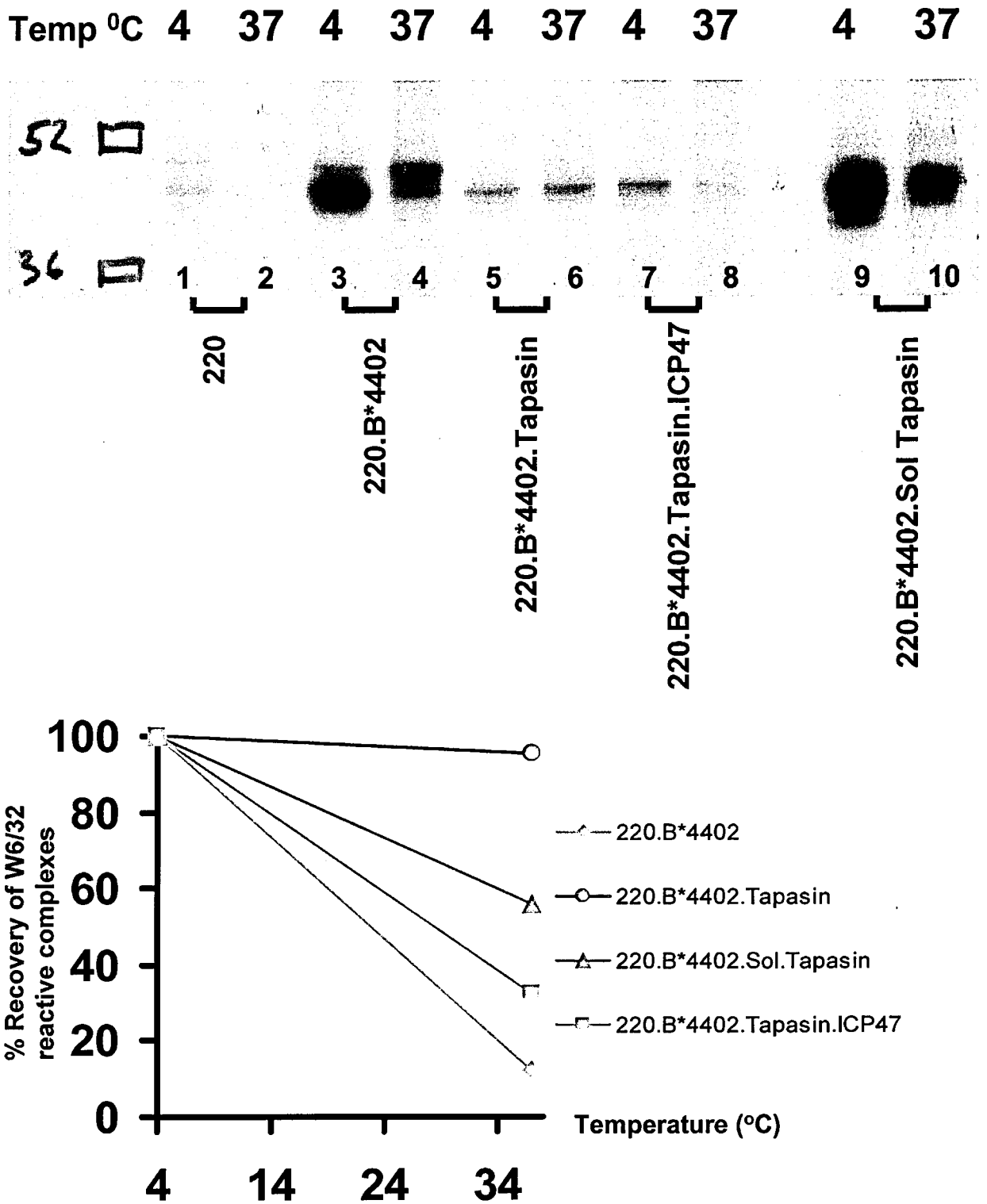
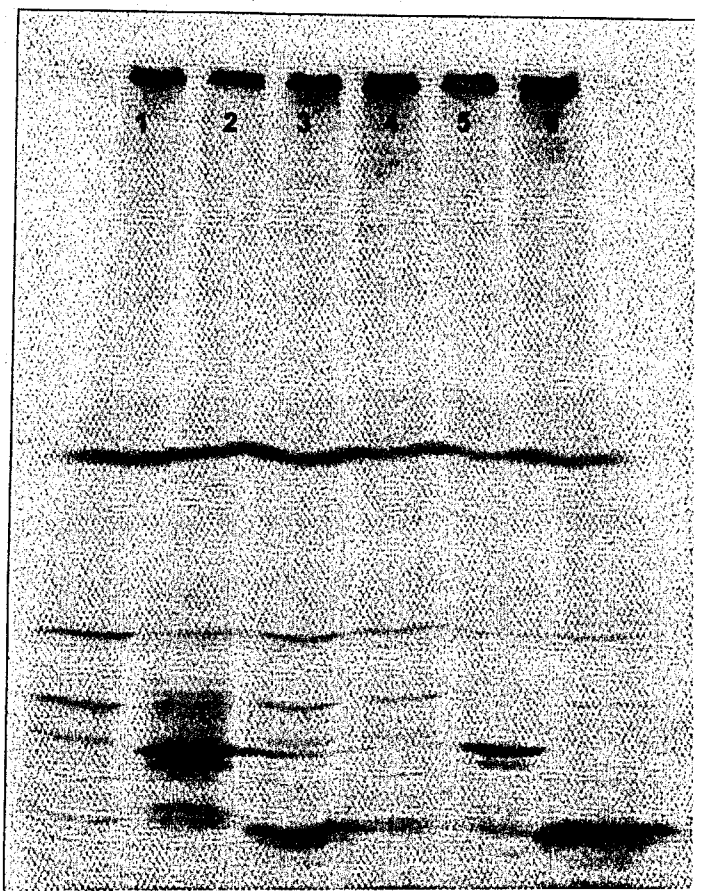


Figure 4.1.1 – Initial thermostability profiles of B*4402

Quantitation of HC signal following the heating of cell lysates from MHC class I competent and incompetent cells.



	C allele (AU)	B allele (AU)	% B allele/Total HC
220	4094	0	**0**
220.B*4402	5193	32812	**86**
220.B*2705	2255	8248	**80**
220.Tapasin	1346	0	**0**
220.B*4402. Tapasin	485	4478	**90**
220.B*2705. Tapasin	767	15407	**95**

Figure 4.1.2 – IEF gel of HC intensity in .220 varlants

was the observation that the complexes loaded in the presence of soluble tapasin were less stable than those seen with full length tapasin (56% versus 95 %). Finally the introduction of a viral TAP inhibitor in the form of ICP47 reduced the stability of the loaded complexes considerably (32% versus 95%). The differences in the recovery of thermostable, peptide loaded complexes were encouraging for the development of a functional assessment of peptide repertoire.

Following this initial analysis of the 220.B*4402 cell line series, a number of modifications were made to the assay to ensure that it could be utilised over a range of temperatures and cell numbers with reproducible accuracy of quantitation. An analysis of cell number is shown in Figure 4.1.3(i) where as few as 1.25 $x10^6$ could be successfully used. An analysis of an increased number of lower temperature points was initially undertaken to probe the less stable complexes. Figure 4.1.3(ii) shows the performance over 7 temperature points from 4°C to 37°C. At higher temperature points (>40°C) aggregates were seen which made quantitation impossible (Fig 4.1.3(iii)). This was previously identified in the work of Tan and colleagues and corrected in a similar manner with the introduction of a high spin speed step following thermal denaturation (Tan et al., 1997). The upper range of thermal denaturation was then assessed and due to the loss of material above 55°C, a highest temperature of 50°C was chosen for further experiments (Fig 4.1.3(iv)). Lastly, the reproducibility of the assay was tested. Pulse label thermostability triplicate experiments were set up and quantitated with a phosphoimager. Even for the poorly stable complexes that

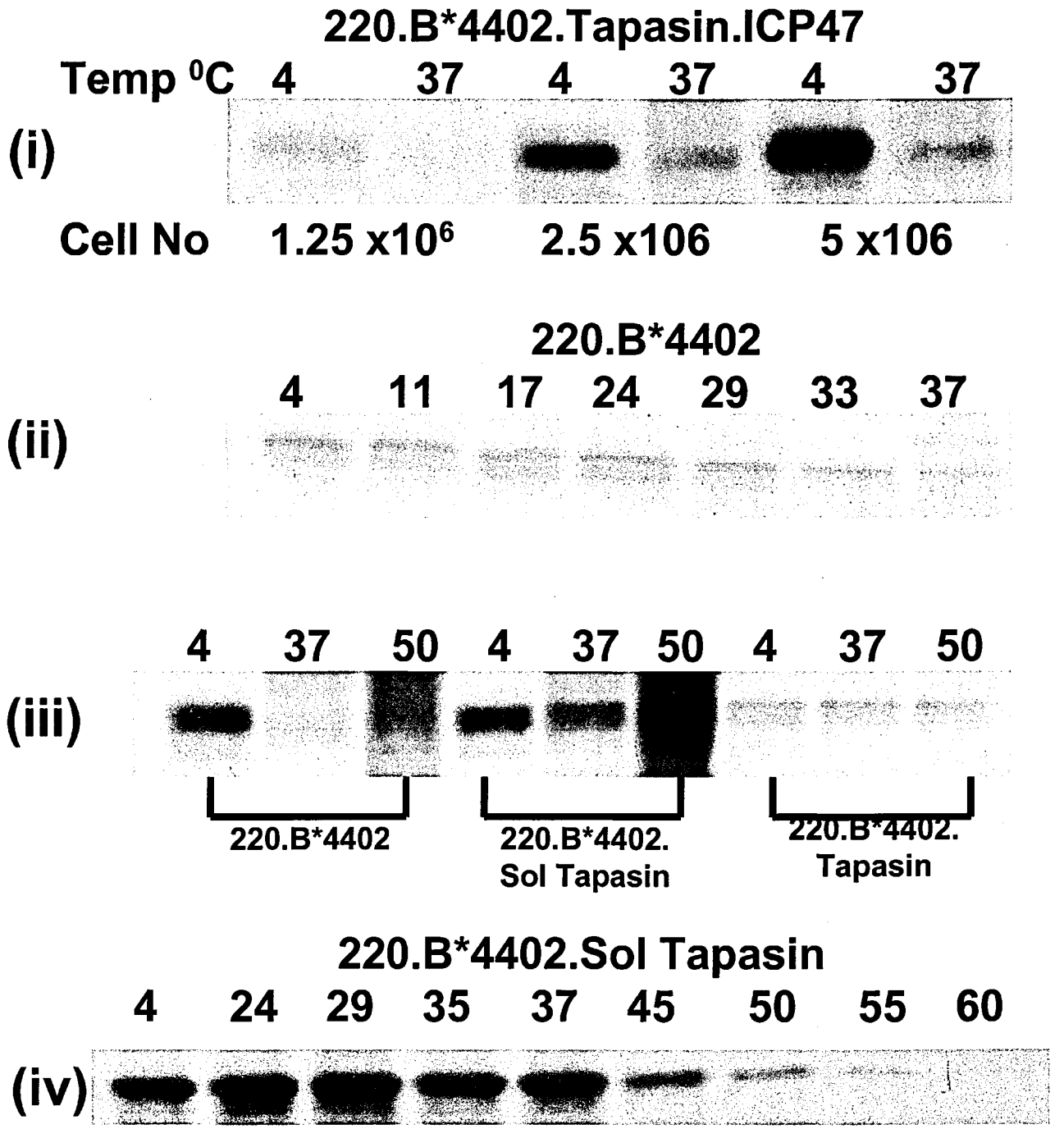


Figure 4.1.3 –Optimisation of the Thermostability assay

The thermostability assay was assessed across different cell numbers and both high and low temperature points.

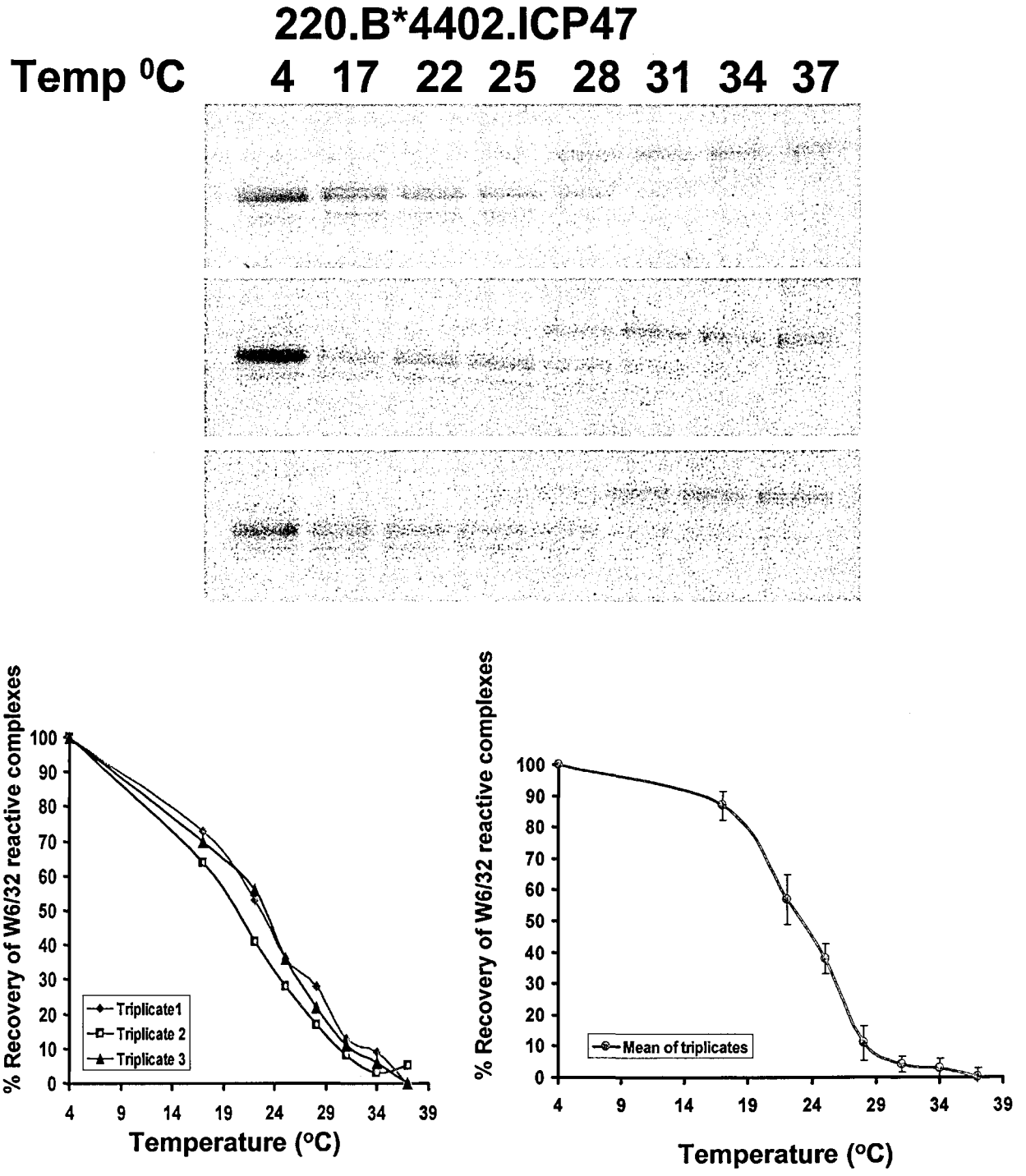


Figure 4.1.4 – Reproducibility of the Thermostability assay

The thermostability assay was assessed in triplicate in cell lines that had exhibited a marked change in the stability of the MHC class I complex.

exhibited a large loss of HC signal over the lower temperature ranges, a low variation about the mean result was seen (Figure 4.1.4). This optimised experimental technique was then used to explore the peptide repertoire of the alleles B*4402, B*2705 and B*4405

4.2. VERIFICATION OF THE RELATIONSHIP BETWEEN MHC CLASS I COMPLEX THERMOSTABILITY AND ITS PEPTIDE CARGO.

Previous studies had correlated the thermostability of a complex with average binding affinity of bound peptide cargo to that complex. In order to determine if this held true for B*4402 and B*2705 I chose a family of B*4402 and B*2705 binding peptides which had been previously characterised (Dedier et al., 2000; DiBrino et al., 1995). These peptides were added to the lysates of cells prior to the thermal denaturation step and their ability to stabilise the complex, compared to a similarly treated lysate in the absence of exogenous peptide. Figure 4.2.1 shows the first experiment that utilised this approach, assessing stability in cells defective in tapasin (.220) or TAP (T2). The B*4402 peptide (SEIDTVAKY) is a self peptide isolated by biochemical elution and the A*0201 peptide (ILKEPVGHGV) is derived from the POL protein of HIV. Two features are seen for both of these alleles. The first is an increased return of W6/32 reactive complexes in the presence of peptide at 4°C (32% for B*4402 and 70% for A*0201). The second feature is an improved stability of the complexes following

(i)

	220.B*4402				T2			
	-Pep		+ 20μM Pep		-Pep		+ 20μM Pep	
	AU	%	AU	%	AU	%	AU	%
4	383	100	505	100 (↑32)	821	100	1399	100 (↑70)
37	94	25	192	38	282	34	1259	90

(ii)

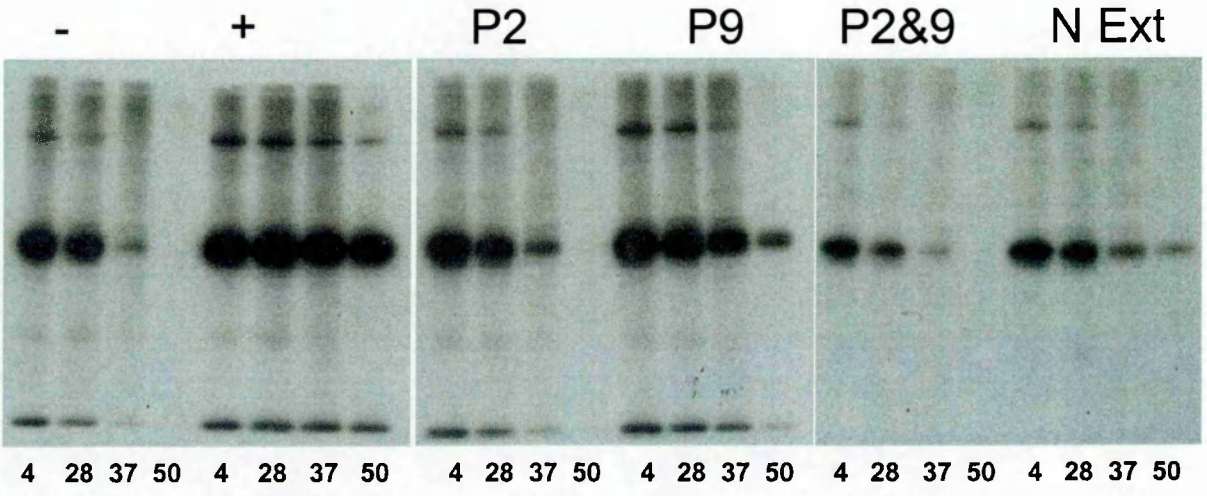
	220.B*4402									
	-		10μM		20μM		100μM		500μM	
	AU	%	AU	%	AU	%	AU	%	AU	%
4	433	100	582	100 ↑34	568	100 ↑31	1496	100 ↑345	1397	100 ↑322
37	48	11	122	21	169	30	951	64	1492	106
50	0	0	104	18	130	23	697	47	1133	81

Figure 4.2.1 – Peptide optimisation assay

(i) Initial peptide addition experiments (20μM) showed an improvement in the thermostabilities of both B*4402 and A*0201. (ii) Increasing the peptide concentration led to an improvement in the thermostability of B*4402, as well as the total number of W6/32 complexes returned.

the addition of peptide (38% for B*4402 and 90% for A*0201). Upon increasing the concentration of peptide from 10uM to 500uM for B*4402 a similar degree of stabilisation could be seen. These initial experiments supported the proposed experimental correlation between thermostability and the peptide cargo of the complex.

To investigate the extent of this correlation, peptides of different affinity were tested. The anchor residues of peptides optimally suited for the B*4402 allele are a glutamic acid (E) at position 2 and a tyrosine (Y) or phenylalanine (F) at position 9. A series of modified peptides were analysed for their hierarchy in stabilising B*4402 within the thermostability assay. Alanine substitutions were introduced at positions P2, P9 as well as an N extended peptide. Figure 4.2.2 shows that the optimal SEIDTVAKY peptide led to a maximal stability of greater than 50% thermostability at 50°C. In comparison the triple alanine N extended 'optimal' peptide had only a 12% return of stable complexes at 50°C. Substitutions at P2 of the peptide appeared to have a greater contribution to the stability of the complex compared those at the P9 position, with a 12 % and 42% stability at 37°C respectively. A second set of B*4402 peptides was analysed that had been previously characterised for optimal binding to B*4402 through radiolabelled β_2m decay experiments.(DiBrino et al., 1995) These peptides were P2 and P9 alanine substituted, N extended and included an aspartic acid substitution at P2. The thermostability profiles of these peptide loaded complexes correlated with the previously determined off rates of β_2m (Figure



	-	+ INDEX SEIDTVAKY	P2 SAIDTVAKY	P9 SEIDTVAKA	P2&9 SAIDTVAKA	N EXT AAASEIDTVAKY
	%	%	%	%	%	%
4	100	100	100	100	100	100
28	68	92	65	70	55	75
37	9	78	12	42	12	27
50	0	62	0	12	0	12

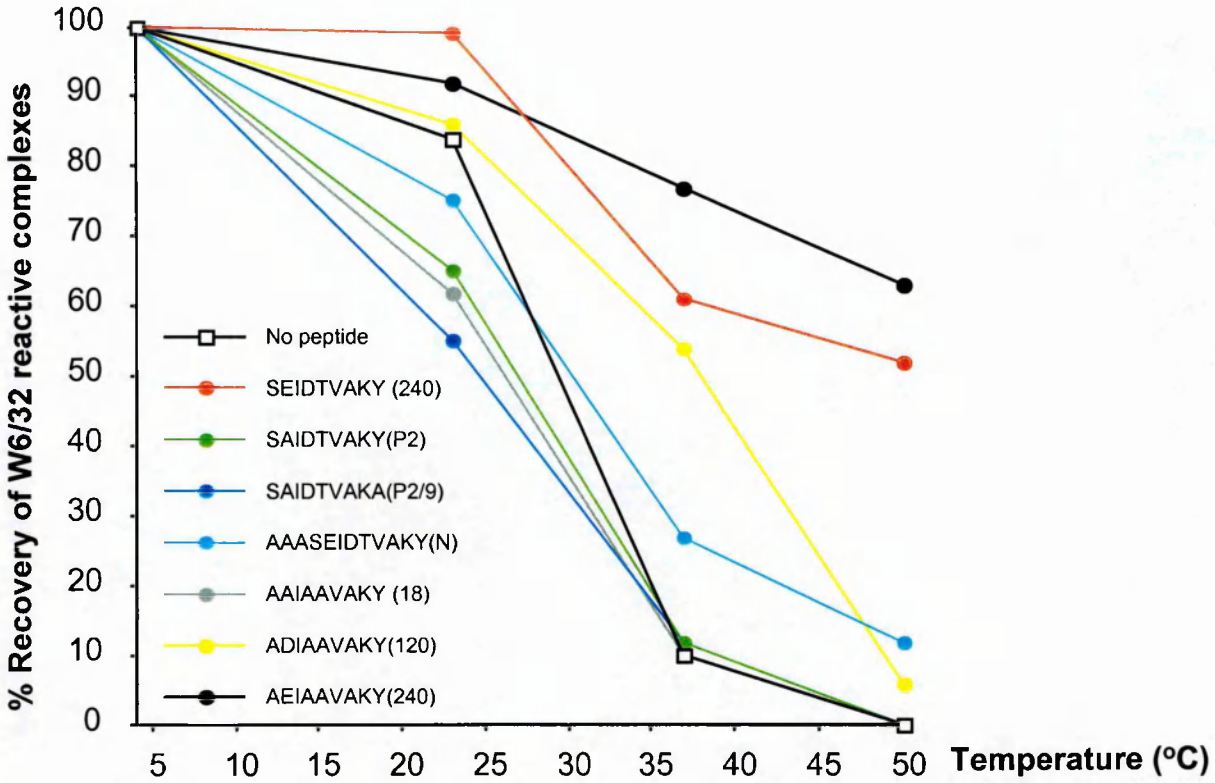
Figure 4.2.2 – B*4402 modified peptide thermostability assay

The anchor residues of the optimal B*4402 peptide (SEIDTVAKY) were modified to establish peptides of lowered affinity for B*4402. These peptides had different thermostability promoting effects upon the B*4402 complexes, with a hierarchy of optimal>N ext>P9 >P2=no peptide.

4.2.3). The optimal peptide AEIAAVAKY had a thermostability profile of 78% at 37°C and 62 % at 50°C compared with 54%:6% for ADIAAVAKY and 10%:0% for the AAIAAVAKY peptide.

This approach was then performed on the B*2705 allele with a series of peptides derived from the HIV-1 gp120 protein. These peptides had been previously characterised for optimal B*2705 thermostability through CD spectroscopy. B*2705 is more promiscuous in the amino acid anchors it will tolerate at positions P2 and P9. The preferred residues are an arginine (R) at position 2 and a lysine (K) at position 9. In comparison to B*4402 which only tolerates tyrosine (Y) and phenylalanine (F) at P9, B*2705 will accept Y/F/M/L/I and H. Due to the absolute requirement of R at position 2, P3 and P9 substituted peptides were used for B*2705. Once again the thermal stability of the peptide occupied complexes correlated with the previously defined peptide hierarchy (Figure 4.2.4). Lastly, this approach was undertaken on the allele B*4405 which differs by one amino acid from B*4402, through a aspartic acid to tyrosine change at position 116 (116D>Y). No previous comparative assays were available on the B*4405 allele, so a number of self and viral peptides were chosen for the thermostability assays (Figure 4.2.5) (Macdonald et al., 2003). Again stability of the poorly loaded complexes isolated from the 220 cell lysates could be markedly improved by addition of all peptides including the SEIDTAVAKY peptide initially identified as a B*4402 binding peptide.

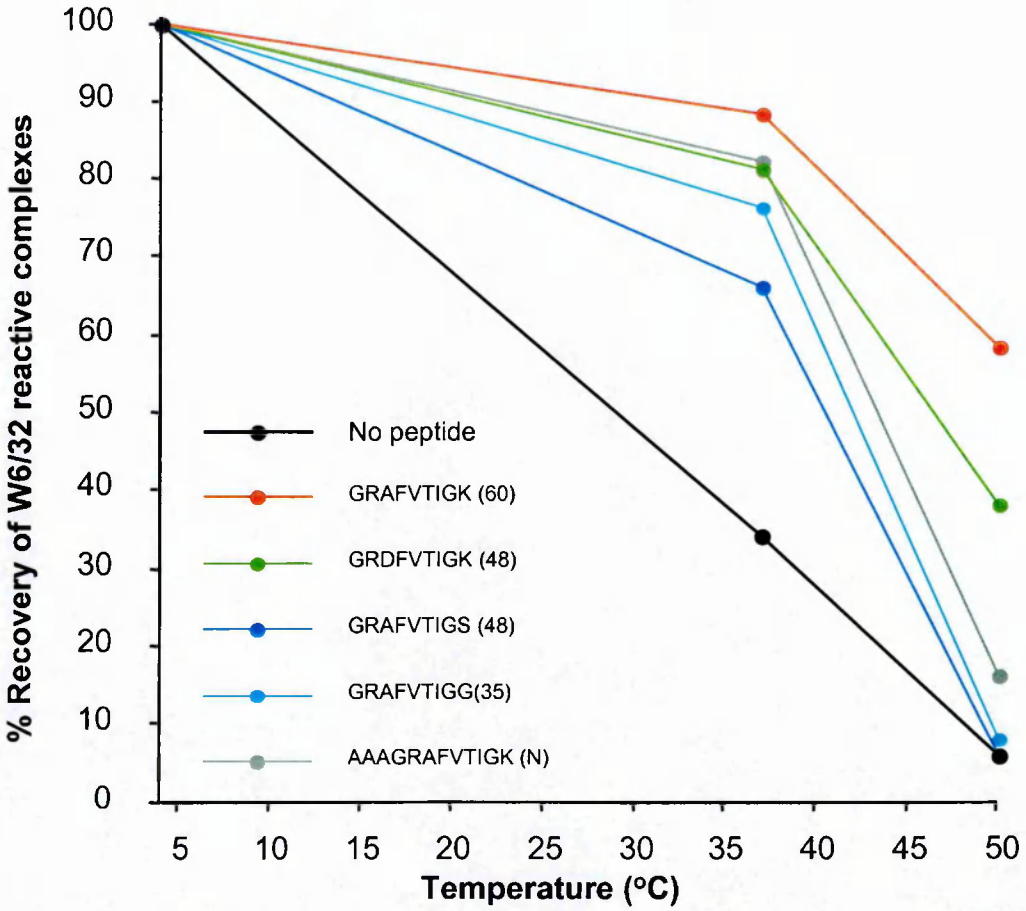
All three alleles showed an enhanced thermostability following the improvement in their peptide cargo through exogenous peptide addition and this improved



	-	AEIAAVAKY (240)	AAIAAVAKY (18)	ADIAAVAKY (120)	AAASEIDTVAKY (N)	240	P2	P2/9
	%	%	%	%	%	%	%	%
4	100	100	100	100	100	100	100	100
28	84	92	62	86	79	99	65	55
37	10	78	10	54	26	61	12	12
50	0	62	0	6	8	52	0	0

Figure 4.2.3 – B*4402 thermostability assay with previously characterised modified peptides.

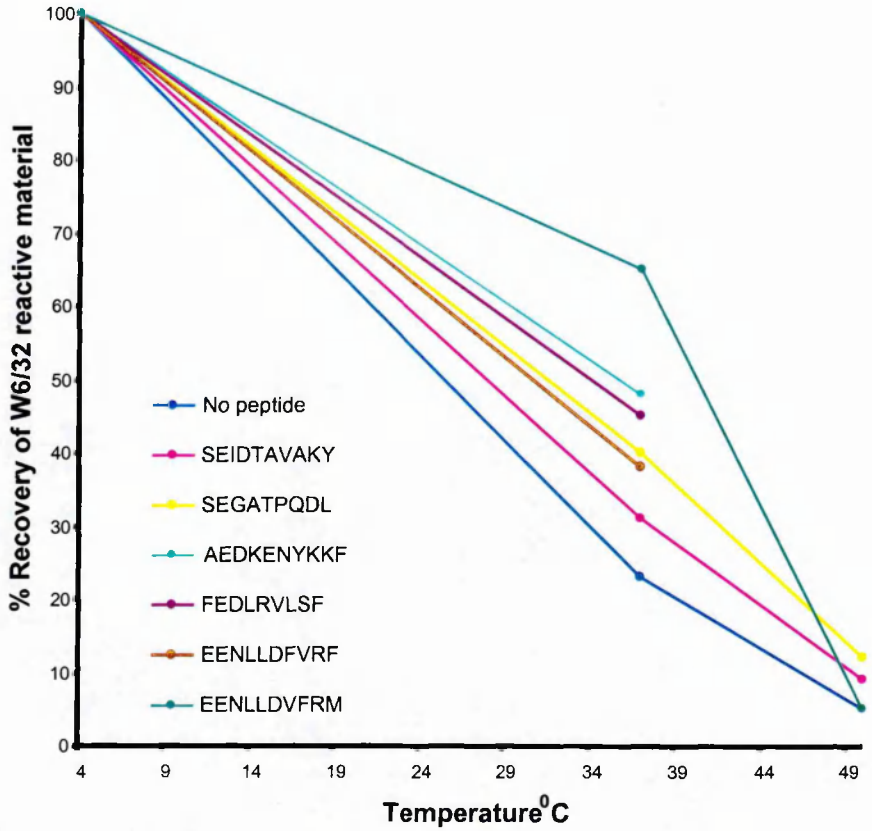
The thermostability assay of B*4402 was undertaken with modified peptides which had been previously characterised according to the half-time for dissociation of B*4402 complexes ($t_{1/2}$). The hierarchy of thermostability follows that of the previously defined $t_{1/2}$.



	-	INDEX GRAFVTIGK (60)	P3 GRDFVTIGK (48)	P9 GRAFVTIGS (48)	P9 GRAFVTIGG (35)	N EXTENDED AAAGRAFVTIGK (N)
	%	%	%	%	%	%
4	100	100	100	100	100	100
37	34	88	81	66	76	82
50	6	58	38	6	8	16

Figure 4.2.4 – B*2705 thermostability assay with previously characterised modified peptides.

The thermostability assay of B*2705 was undertaken with modified peptides which had been previously characterised by CD spectroscopy. The hierarchy of thermostability was comparable to the previously documented thermal denaturation, monitored by CD spectroscopy.



	-	SEIDTVAKY	SEGATPQDL	AEDKENYKKF	FEDLRVLSF	EENLLDFVRF	EEELLDVFRM
	%	%	%	%	%	%	%
4	100	100	100	100	100	100	100
37	23	31	40	48	45	38	65
50	5	9	12	ND	ND	ND	5

Figure 4.2.5 – B*4405 thermostability assay with previously characterised modified peptides.

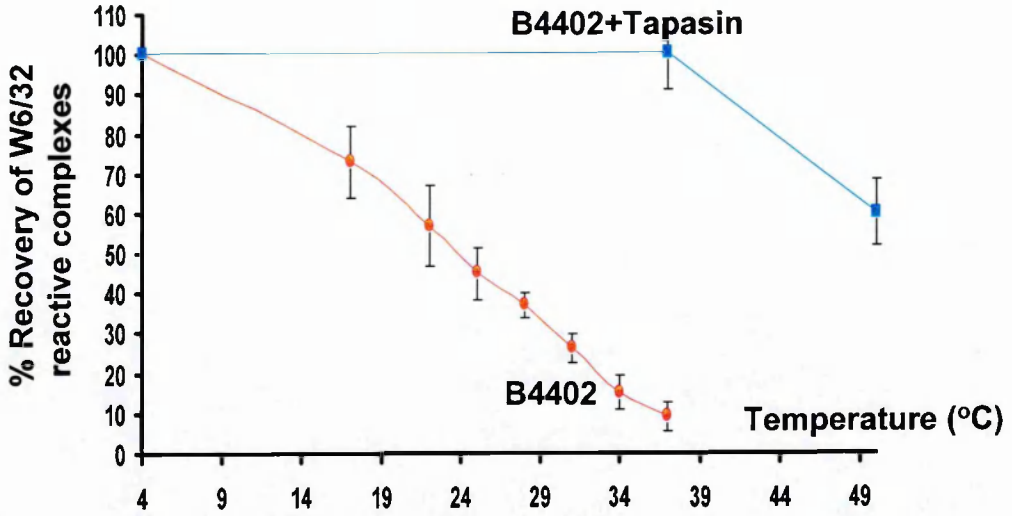
The thermostability assay of B*4405 was undertaken with selected peptides (previously identified by elution). The B*4402 binding peptide SEIDTVAKY was also used.

thermostability correlated well with the known affinities of the peptides. The proof of principle experiments permitted the subsequent analysis of intracellular bulk MHC class I complex thermostability profiles, as a measure of their optimal peptide occupancy within different cell lines. Thus, we felt that the thermostability of a MHC class I complex was a good indicator of the average peptide cargo affinity and this underpins the following experiments.

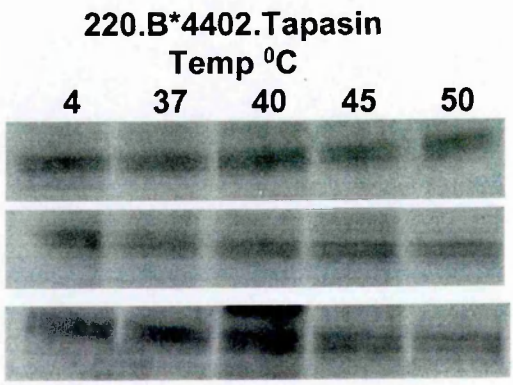
4.3 STUDIES ON THE TAPASIN DEPENDENCE OF HLA-B*4402 AND B*2705

4.3.1 B*4402 AND B*4402.TAPASIN THERMOSTABILITY

The following experiments determined the thermostability profiles of newly synthesised B*4402 complexes expressed in the presence and absence of tapasin. A cohort of newly synthesised complexes, labelled for 30 minutes with ^{35}S, were analysed for their thermostability characteristics over a range of temperatures. Figure 4.3.1.1 shows the poor stability of B*4402 complexes expressed in the absence of tapasin with only 45% of complexes surviving at 25°C and 9% at 37°C. This is consistent with B*4402 molecules having bound a spectrum of suboptimal ligands in the absence of tapasin. This suboptimal peptide occupancy is mirrored by the poor cell surface expression of B*4402 in .220 cell lines. In contrast, B*4402 complexes loaded in the presence of tapasin are all thermostable at 37°C, with the majority stable at 50°C. This improvement



B*4402 Tapasin	Triplicate 1	Triplicate 2	Triplicate 3	Mean
	% of 4°C	% of 4°C	% of 4°C	
4	100	100	100	100
37	108	91	103	100
50	63	51	67	60



B*4402	Triplicate 1	Triplicate 2	Triplicate 3	Mean
	% of 4°C	% of 4°C	% of 4°C	
4	100	100	100	100
17	73	82	64	73
22	69	50	53	57
25	52	39	45	45
28	38	34	40	37
31	28	23	30	26
34	10	16	18	15
37	7	13	7	9

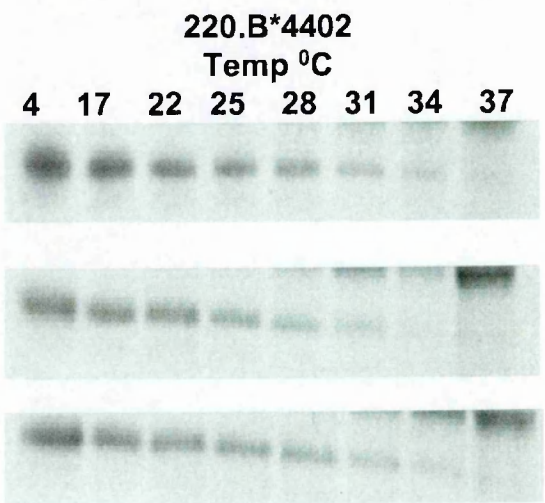
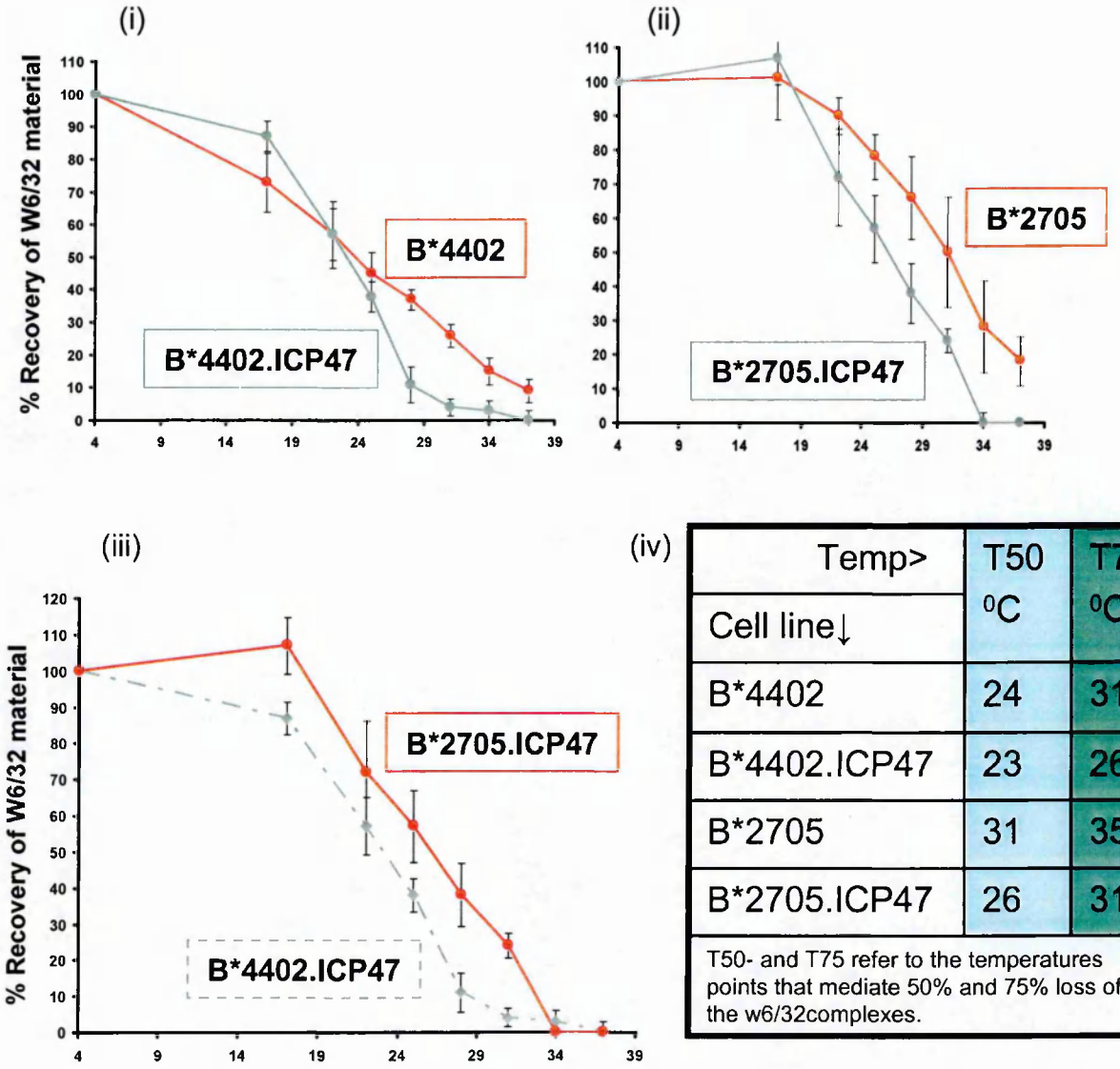


Figure 4.3.1.1 – Thermostability of B*4402 +/- Tapasin.

Triplicate experiments of B*4402 +/- tapasin were quantified and plotted.

in thermostability indicates that the complexes have bound peptides that have an affinity are at least as high as the index peptides characterised in Figure 4.2.3. This improvement in thermostability is mirrored by an increase in the cell surface appearance of B*4402 complexes.

The poor thermostability of B*4402 complexes suggested that they could either be empty complexes or loaded with suboptimal peptide ligands. To test this question further I examined the thermostability profile of B*4402 complexes in the presence of the viral TAP inhibitor ICP47 (Fig 4.3.1.2 and Fig 4.3.1.3). The thermostability experiments were performed 3 times, and showed a further deterioration in the thermostability profile of B*4402 complexes loaded in the absence of a TAP dependent peptide supply. This was most marked after the 25°C temperature point with comparable stabilities up until this point. These observations are important for 2 reasons. The first is that B*4402 complexes are less stable in the presence of ICP47, indicating that in the presence of a peptide supply B*4402 complexes are probably not empty. This implies that the B*4402 complexes in tapasin deficient cells have either bound and lost peptide with a consequential improvement in thermostability of the empty complex (but which still remains below the 'quality standard' to bypass ER quality control) or are occupied with low affinity peptides that cannot be exchanged. The second observation is that this quantified change in the thermostability between the 2 complexes, establishes a minimum thermostability that permits cell surface expression.



Temp> / Cell line↓	T50 ^{0}C	T75 ^{0}C
B*4402	24	31.5
B*4402.ICP47	23	26.5
B*2705	31	35
B*2705.ICP47	26	31

T50- and T75 refer to the temperatures points that mediate 50% and 75% loss of the w6/32complexes.

Figure 4.3.1.2 – Thermostability of B*4402 and B*2705 +/-ICP47.

Triplicate experiments of B*4402 and B*2705 +/- ICP47 were quantified and plotted.
(i) Comparison of B*4402 in the presence and absence of a TAP mediated peptide supply
(ii) Comparison of B*2705 in the presence and absence of a TAP mediated peptide supply
(iii) Comparison of the thermostability of B*4402 and B*2705 in the absence of a TAP mediated peptide supply.
(iv) There is a greater stability of B*2705 compared to B*4402 in the presence of ICP47 and in the absence of tapasin.

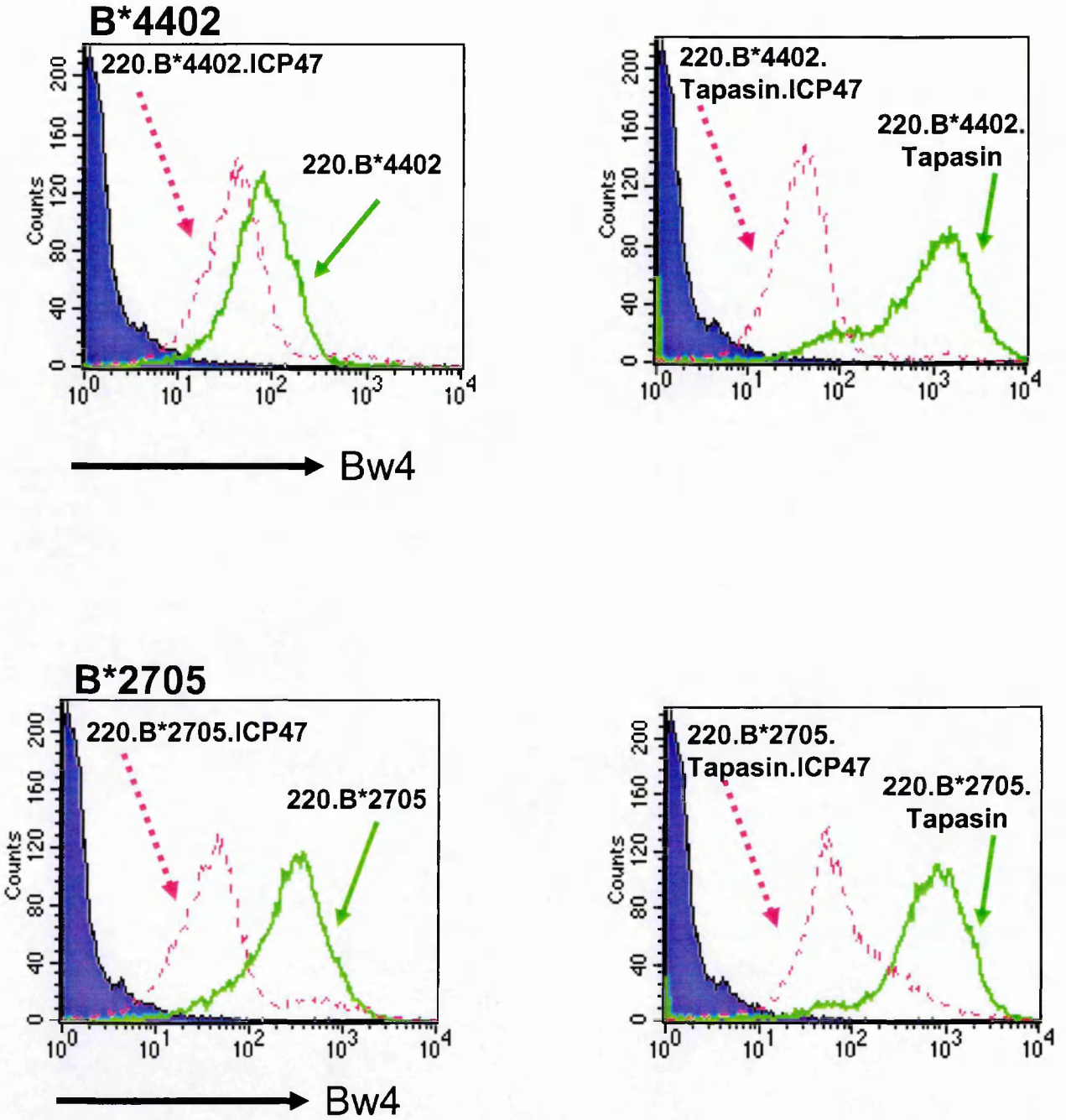
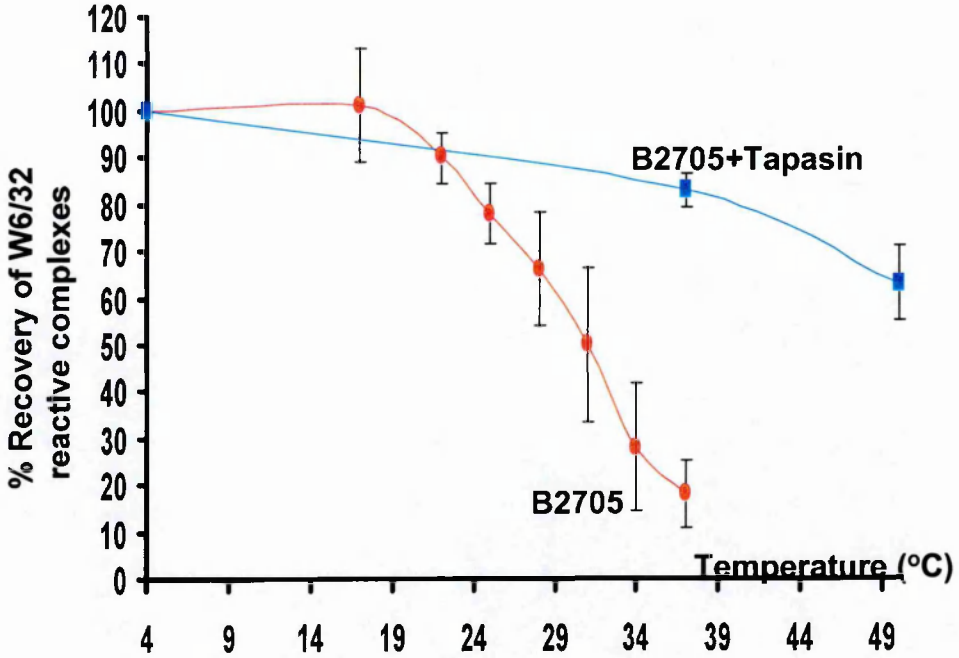


Figure 4.3.1.3 – FACS of B*4402 ICP47 and B*2705 ICP47 +/- Tapasin.

Following the introduction of ICP47 into the 220.B*4402 cell line the MFI falls from 89 to 67.
For the 220.B*4402.Tapasin cell line the MFI falls from 1160 to 54.
In the 220.B*2705 cell line the ICP47 reduces the MFI from 337 to 98
In the 220.B*2705.Tapasin cell line the MFI falls from 817 to 136

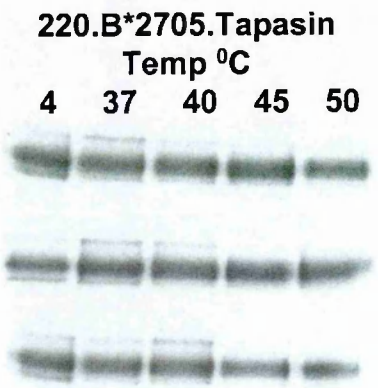
4.3.2 B*2705 AND B*2705.TAPASIN THERMOSTABILITY

B*2705 was originally reported as being tapasin independent, showing comparable steady state cell surface expression in the presence or absence of tapasin (Peh et al., 1998). Surprisingly the newly synthesised cohort of B*2705 complexes were very thermolabile in the absence of tapasin (Figure 4.3.2.1). Only 18% of complexes survived heating to 37°C, a figure not that different to the 9% of B*4402 complexes seen at this temperature. In contrast the B2705 complexes loaded in the presence of tapasin showed excellent thermostability with nearly all complexes stable at 37°C. This was a clear indication that this allele was dependent on tapasin for the optimal loading of peptides: a result that was initially difficult to reconcile with the steady state cell surface data. Upon the introduction of ICP47 a further reduction in the thermostability of the complexes was seen (Figure 4.3.1.2 and Figure 4.3.1.3). However the thermostability of the B*2705 complexes was always greater than that of the B*4402 complexes, even in the absence of peptide and tapasin. Although this might be interpreted as evidence of a greater stability of the 'empty' form of B*2705 compared to B*4402 consideration should be given to the availability of TAP independent peptides. The ability of B*2705 to bind such peptides has been previously demonstrated in transfection experiments upon the TAP deficient T2 cell line (Anderson et al., 1993).



B*2705 Tapasin	Triplicate 1	Triplicate 2	Triplicate 3	Mean
	% of 4°C	% of 4°C	% of 4°C	
4	100	100	100	100
37	79	84	86	83
50	55	71	62	63

B*2705	Triplicate 1	Triplicate 2	Triplicate 3	Mean
	% of 4°C	% of 4°C	% of 4°C	
4	100	100	100	100
17	90	100	114	101
22	86	88	96	90
25	82	81	70	78
28	52	75	70	66
31	39	69	43	50
34	27	42	15	28
37	17	26	12	18



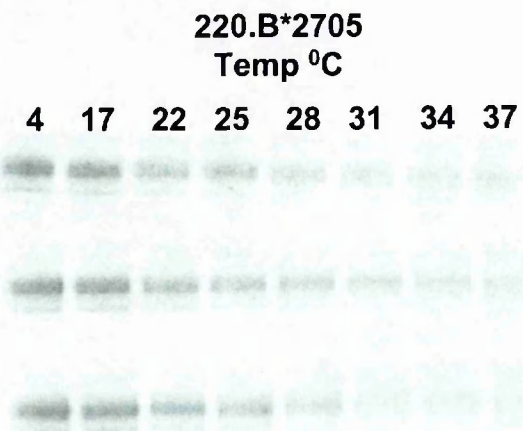


Figure 4.3.2.1 – Thermostability of B*2705 +/- Tapasin.

4.4 COMPARISON OF THE THERMOSTABILITY OF CELL SURFACE AND INTRACELLULAR B*2705

The finding that the early cohort of B*2705 complexes assembled in the absence of tapasin were less thermostable than those assembled in the presence of tapasin appeared to be inconsistent with the cell surface expression of B*2705. I compared the thermostability of cell surface radioiodinated B*2705 complexes with a ^{35}S labelled intracellular cohort of newly synthesised B*2705 complexes. Figure 4.4.1 shows that in the presence of tapasin the early cohort of intracellular molecules are as stable as the cell surface molecules. In contrast only the cell surface B*2705 complexes were stable in the tapasin negative cell line with the intracellular pool having marked thermoinstability. This suggested that B*2705 is dependent upon tapasin although this property is less obvious when the steady state cell surface pool of molecules is analysed. Furthermore it raised the question of how the previously unstable early cohort of B*2705 molecules attain eventual stability. One possibility was that such complexes could optimise their peptide cargo allowing the eventual cell surface pool to have an enhanced thermostability. I tackled this latter possibility by further developing the assay to follow complexes over time

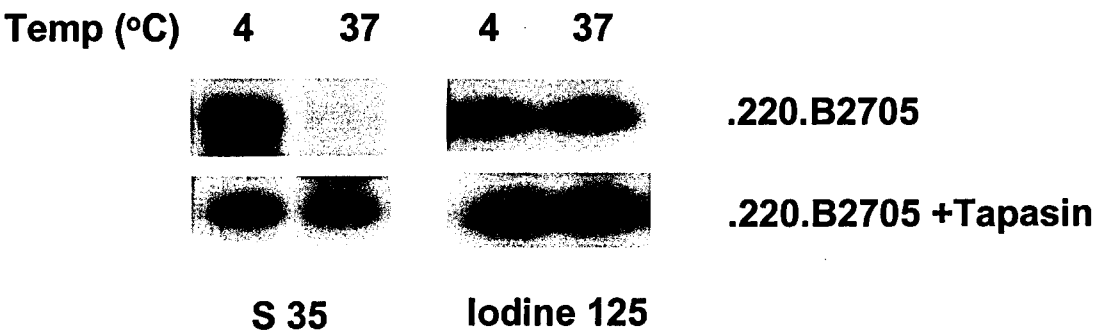


Figure 4.4.1 – Thermostability comparison of cell surface and intracellular B*2705 complexes +/- Tapasin

B*2705 complexes in tapasin deficient cells are stable at the cell surface but are less stable in the ER. In the presence of Tapasin the B*2705 complexes are equally stable in the ER and at the cell surface.

4.5 TIME DEPENDENT OPTIMISATION OF MHC CLASS I ALLELES

4.5.1 A PULSE CHASE THERMOSTABILITY ASSAY FOR AN ASSESSMENT OF THE CHANGING PEPTIDE REPERTOIRE OF MHC CLASS I ALLELES OVER TIME.

To further examine the discrepancy between the thermostability of an early cohort of MHC class I complexes and the steady state cell surface pool, I modified the thermostability assay to allow for the characterisation of a cohort of MHC class I molecules over time. Figure 4.5.1.1 shows the initial experiment upon the allele B*2705 in the absence of tapasin. A five minute pulse label was now utilised and the complexes were analysed at 10, 30, 60 and 120 minutes. This initial experiment was interesting for a number of reasons. Firstly it showed that between 10 and 30 minutes there was on-going assembly of MHC class I HC with β_2m to form W6/32 reactive complexes and that between 30 and 60 minutes there was a plateau of 4°C stable complexes. Most interestingly the amount of 37°C stable material increased between 30 and 60, and 60 to 120 minutes. There was also a smaller rise in the 50°C stable material over this time. As the 4°C material was stable, this figure could be used as the denominator in deriving a percentage stability (% stability) at both 37°C and 50°C over time. When this pulse chase was extended to 180 minutes no further improvement in the number of 37°C or 50°C complexes was seen (Figure 4.5.1.2). However

(i)

	220.B*2705							
	10 mins		30 mins		60 mins		120 mins	
	AU	%	AU	%	AU	%	AU	%
4	473	100	604	100	683	100	615	100
37	148	31	205	34	373	55	509	83
50	50	11	116	19	177	26	191	31

(ii)

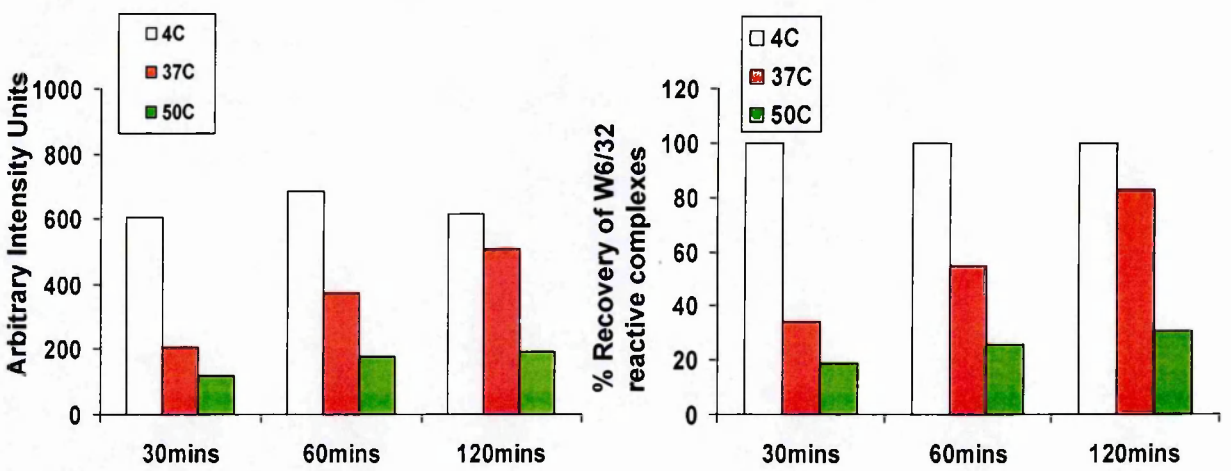


Figure 4.5.1.1 – Pulse chase Thermostability assay of B*2705 (2hrs)

A 2 hour pulse chase of B*2705 was undertaken with thermostability profiles determined at 30,60 and 120 minutes.

(i) Quantification of the HC signal at each time and temperature point shows an increase in the signal over time.

(ii) A graphical representation of the data illustrates both the change in total HC recovered (AU) and the % stability at each temperature and time point .This % stability relates to the total material recovered at 4°C.

(i)

	220.B*2705							
	30 mins		60 mins		120 mins		180 mins	
	AU	%	AU	%	AU	%	AU	%
4	811	100	750	100	672	100	572	100
37	401	49	447	60	517	77	495	87
50	95	12	182	24	316	47	256	46

(ii)

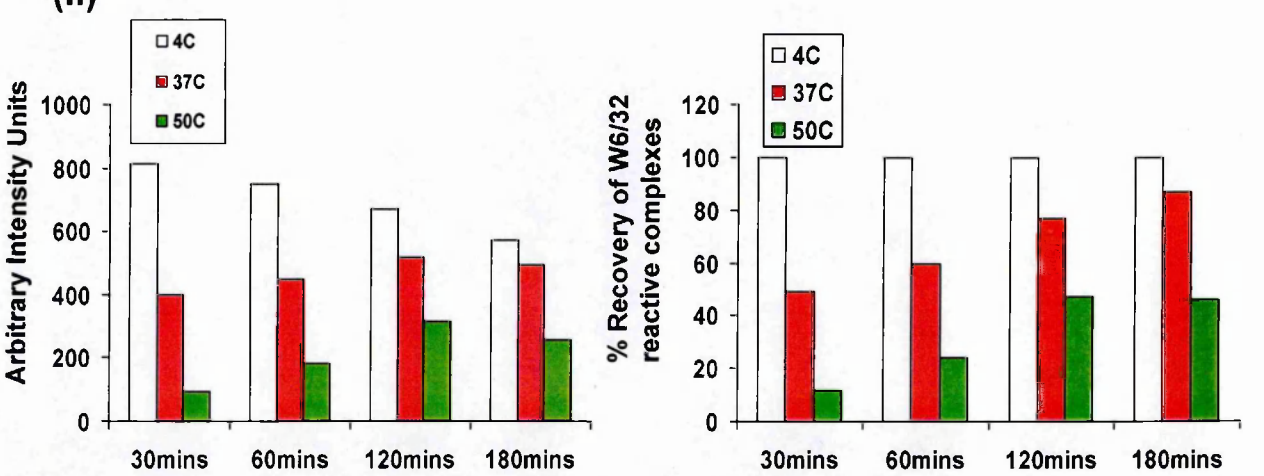


Figure 4.5.1.2 – Pulse chase Thermostability assay of B*2705 (3hrs)

A 3 hour pulse chase of B*2705 was undertaken with thermostability profiles determined at 30, 60, 120 and 180 minutes.

(i) Quantification of the HC signal at each time and temperature point shows a decay in the HC signal that is most marked between 2- 3 hours. The loss of material is seen in 4°C,37°C and 50°C complexes.

(ii) A graphical representation of the data illustrates both the change in total HC recovered (AU) and the % stability at each temperature and time point .This % stability relates to the total material recovered at 4°C. When there is loss of 4°C material a 'false' increase in the % stability may be seen.

during the longer pulse chase there was some loss of the 4°C material which 'artificially' increased the proportion of complexes appearing to be 37°C and 50°C. For this reason the results of these and all further experiments were expressed as arbitrary units of HC (AU) and proportion of stable complexes.

A further modification to the assay was the introduction of an endoglycosidse H digestion step following the heating and immunoprecipitation steps. This would provide an analysis of the newly synthesised cohort of molecules at the pre medial golgi and post medial golgi stages of their trafficking. Additionally it would provide an assessment of the trafficking characteristics of complexes with differing thermostabilities and hence peptide repertoires. Figure 4.5.1.3 shows the initial experiment on 220.B*2705 over a 2 hour pulse chase. This experimental approach provides a wealth of information. It is possible to enumerate:

1) Total time dependent optimisation (figures in italics)
2) Total time dependent optimisation in the endo R cohort (figures in bold/blue)
3) Total time dependent optimisation in the endo S cohort (figures in bold/green)
4) Relationship between the different thermostable cohorts (**>4°C<37°C** and **>37°C<50°C** and **>50°C)** as they move between the endo S – to endo R fractions
5) Pulse chase assessments on 4°C material (typical pulse chase calculation)
6) Pulse trafficking characteristics of the different thermostable cohorts.

(i)

NO ENDO H	220.B*2705 Endo H +/-					
	30 mins		60 mins		120 mins	
	AU	%	AU	%	AU	%
4	811	**100**	750	**100**	672	**100**
37	401	**49**	447	**60**	517	**77**
50	95	**12**	182	**24**	316	**47**

(ii)

ENDO H	220.B*2705 Endo H +/-					
	30 mins		60 mins		120 mins	
	AU	%	AU	%	AU	%
4 Total	822	*100*	771	*100*	678	*100*
Endo R	404	**100**	476	**100**	558	**100**
Endo S	418	**100**	295	**100**	120	**100**
37 Total	366	*44*	530	*69*	567	*84*
Endo R	224	**55**	417	**80**	492	**88**
Endo S	141	**33**	113	**38**	75	**62**
50 Total	129	*16*	191	*25*	247	*36*
Endo R	93	**23**	150	**32**	199	**37**
Endo S	36	**9**	42	**14**	48	**46**

Figure 4.5.1.3 – Pulse chase Thermostability assay of B*2705 +/- Endo H digestion.

The thermostability assay was undertaken following a true or mock Endoglycosidase H digestion. As before, the improving thermostability profile is seen in the absence of endo H digestion (i). In the presence of endo H (ii) the addition of the endo S and endo R fractions gives a similar result to (i).

Figure 4.5.1.3 shows a similar result to Figure 4.5.1.2 with an increase in the 37°C and 50°C stable complexes over the two hour time period. In addition, the endoglycosidase arm of the experiment shows comparable results when the endo sensitive (endo S) and resistant (endo R) fractions are added together. Figure 4.5.1.4 illustrates this, showing that B*2705 Endo H +Total experiment is similar to the preceeding experiments without digestion. In considering the individual cohorts within the pulse chase thermostability analysis of the endo H positive experiment, 2 points can be made.

1) The first is the clear difference in the 4°C complexes over time, with a decrease in the endo S cohort and an increase in the endo R cohort. The reduction in the endo S cohort could be secondary to a transfer of such complexes out of the ER and through the medial golgi to become endo R complexes between time points or a removal of such complexes from the ER to a degradative pathway (i.e. ERAD).
2) When the 37°C complexes are considered, a continuing increase in the endo R fraction is seen. This is marked for the 50°C stable complexes whose number in the endo S pool in very low at all time points but continues to increase overtime in the endo R pool.

Figure 4.5.1.5 attempts to further define the origin of the 37°C and 50°C complexes. In particular, whether they are all delivered from the endo S pool as 37°C or 50°C stable complexes or if they are derived from the pre-existing >4°C but < 37°C cohort from within the endo R pool. All the AU figures within these graphs are tabulated in Figure 4.5.1.3. From the 4°C, 37°C and 50°C figures we

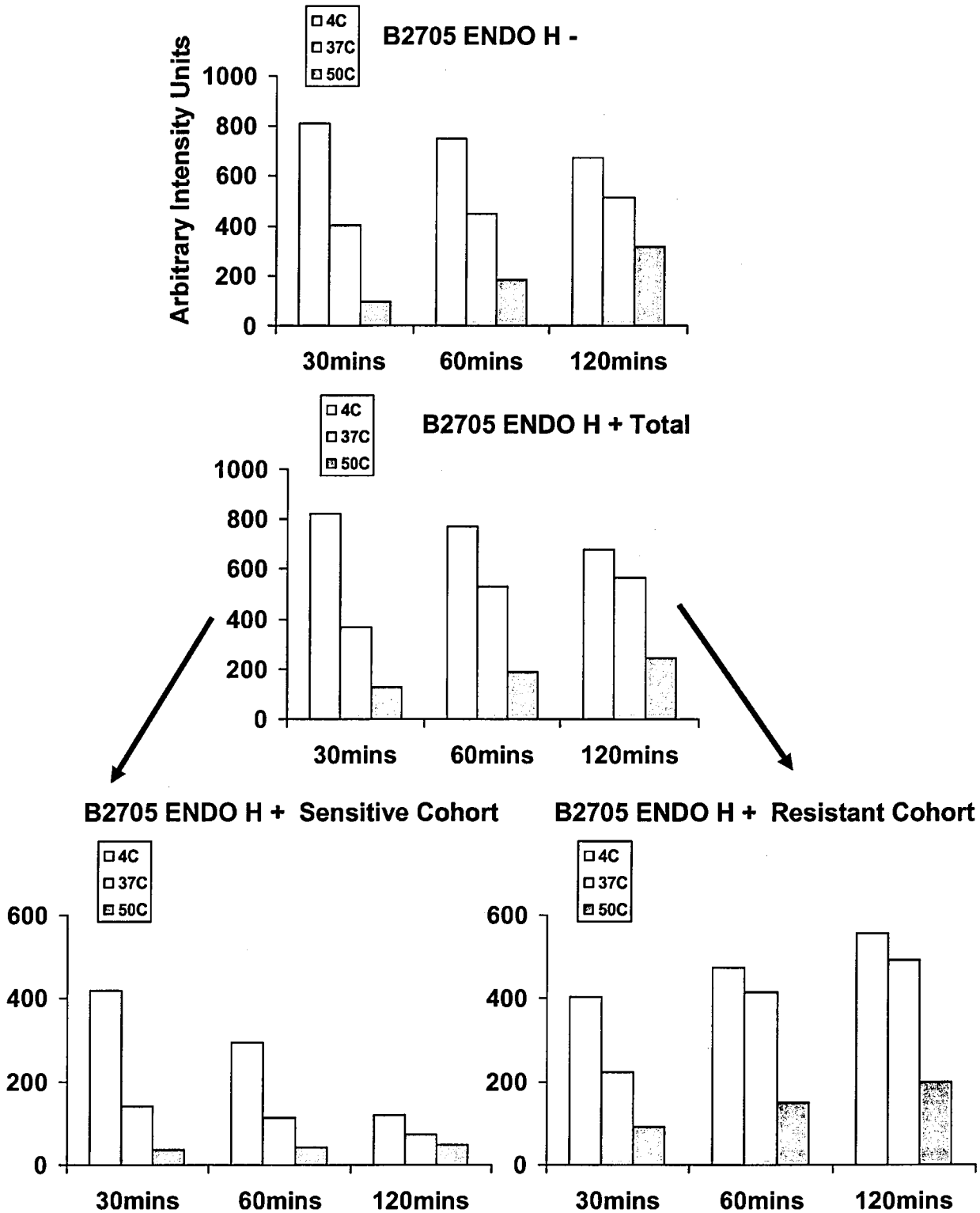


Figure 4.5.1.4 – Cohort analysis of the pulse chase Thermostability assay of B*2705 +/- Endo H digestion.

The endo H + experiment allows for the representation of 2 cohorts of class I molecules. The endo S cohort are distinct and may enter the endo R cohort (or ERAD) whilst the endo R cohort may undergo further 'autonomous' change (post ER) and be modified by influx of endo S complexes between time points.

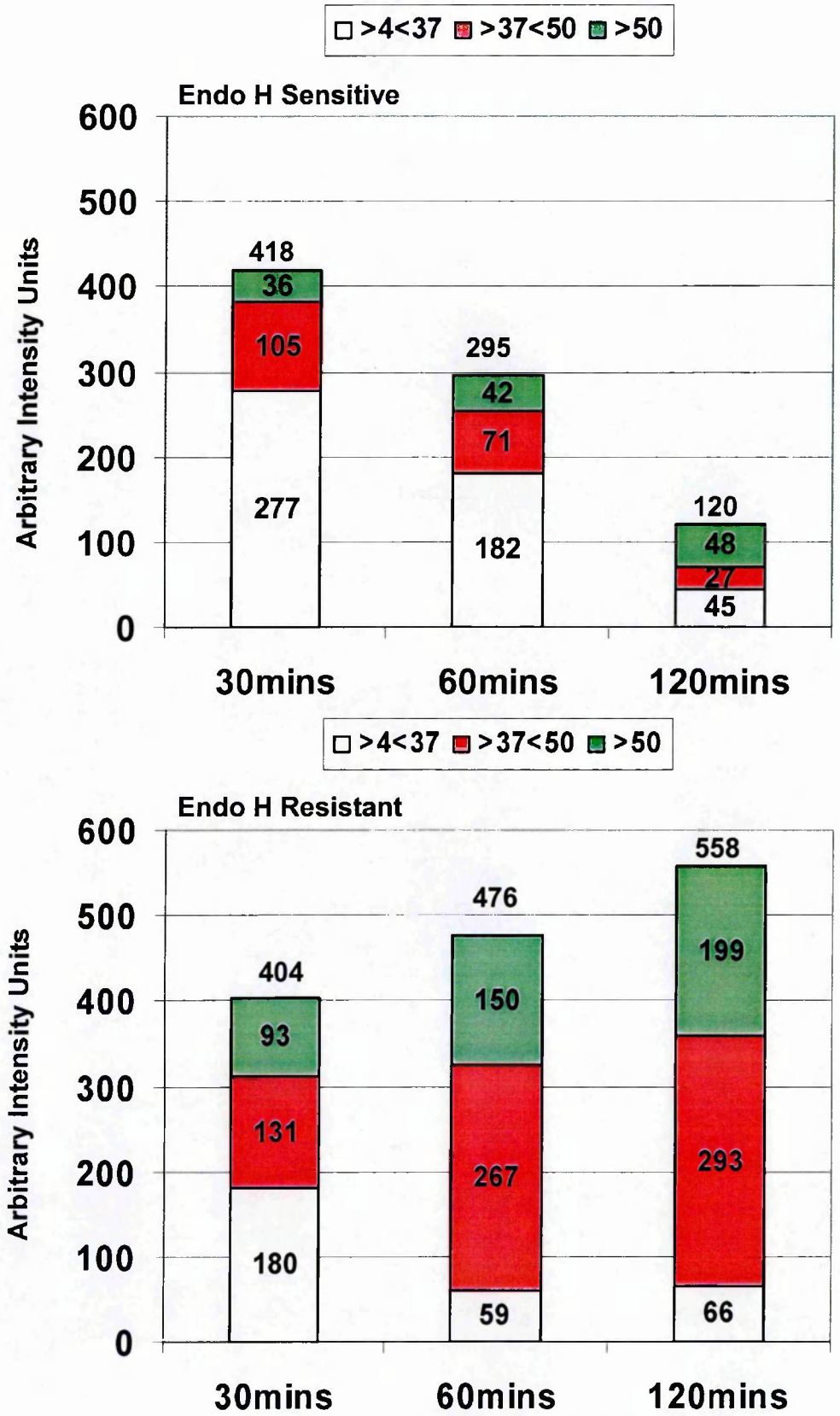


Figure 4.5.1.5 – Evaluation of the origin of endo R thermostable complexes within the thermostability assay of B*2705 +/- Endo H digestion.

can derive the absolute number of some other thermostable cohorts. For example if we consider the 30 minute endo S cohort we can define:

>4°C but <37° cohort (4°C cohort (418) -37°C cohort (141) = 277 (WHITE)

>37°C but <50°C cohort (37°C cohort (141) - 50°C cohort (36) = 105 (RED)

If we then consider the endo R fraction between the 30 to 60 minute time period, the >37°C stable material increased from 224 AU to 417 AU, a difference of 193 AU. The 'loss' from the endo S pool between these times is only 123 AU (418-295) suggesting that some of the increase in 37°C stable material may be generated within the endo R pool. Interestingly, when the >4°C but <37°C endo R material (WHITE) is assessed at these time periods, a fall of 122 AU (180 – 59AU) (122 AU) is seen. This loss is within a post ER cohort at a time when there is little total loss from the experiment (822-771AU) and so it is tempting to speculate that this is due to conversion of this material a 37°C stable pool within the endo R fraction. This 'autonomous change' would supplement the incoming endo S material, in enhancing the overall thermostability characteristics of the endo R pool. Between 60 and 120 minutes the endo S >4°C material fell from 295 to 120 (175 AU) whilst the increase in the endo R pool was only 82 AU (558-476). This suggests that during this time period some of the poorly loaded complexes in the endo S pool were lost from the experiment. As these complexes are not seen in the trafficking populations it is probable that they have been degraded. It must be stressed that all these approximations are considered in light of this assay being a semi quantitative assay (at best) but they do permit

a viewpoint upon the qualitative nature of newly synthesised MHC class I complexes that has not been previously possible.

Figure 4.5.1.6 shows the comparative pulse chase kinetics of the differing thermostability cohorts. The trafficking half life of B*2705 is 30 minutes at 4°C. This is similar to that of B*2705 expressed in the presence of tapasin (see later). Surprisingly the 37°C and 50°C stable complexes appeared to have a faster trafficking rate with 61% of 37°C stable and 72% of 50°C stable material endo R at 30 minutes, compared to 49% of the total cohort. It is possible that this reflects the faster egress of more stable complexes from the ER compared to the bulk 4°C stable pool. Given the improved stability of complexes loaded in the presence of tapasin it would be predicted that these complexes would demonstrate a faster trafficking rate than those loaded in tapasin deficient cells. However, this is not the case for reasons that are not entirely clear.
These initial experiments provided the impetus to analysis the pulse chase thermostability profiles of B*4402 and B*2705 in tapasin competent and incompetent cell lines.

4.5.2 TAPASIN CATALYSES THE TIME DEPENDENT OPTIMISATION OF THE B*4402 PEPTIDE REPERTOIRE.

Figure 4.5.2.1 shows the pulse chase thermostability results for B*4402 expressed in the presence and absence of tapasin. The first clear difference is

220.B*2705	30 mins		60 mins		120 mins	
4°C	AU	% Endo H R/S	AU	% Endo H R/S	AU	% Endo H R/S
Endo R	404	**49**	476	**62**	558	**82**
Endo S	418	51	295	38	120	18
Total	822		771		678	

220.B*2705	30 mins		60 mins		120 mins	
37°C	AU	% Endo H R/S	AU	% Endo H R/S	AU	% Endo H R/S
Endo R	224	**61**	417	**79**	492	**87**
Endo S	141	39	113	21	75	13
Total	366		530		567	

220.B*2705	30 mins		60 mins		120 mins	
50°C	AU	% Endo H R/S	AU	% Endo H R/S	AU	% Endo H R/S
Endo R	93	**72**	150	**79**	199	**81**
Endo S	36	28	42	21	48	19
Total	129		191		247	

Figure 4.5.1.6 – Pulse chase characteristics of different thermostable B*2705 cohorts.

Typical pulse chase calculations enumerate the fraction of total complexes that have achieved endo R status at each time point. This is typically undertaken with all the complexes kept at 4°C. The first table (i) shows this approach at the 30, 60 and 120 minute time points. Tables (ii) and (iii) analyse the fractions that have attained Endo R status within the more thermostable cohorts.

the loss of 4°C stable material over time in the absence of tapasin. This loss is probably due to the dissociation of β_2m following loss of low affinity peptides with subsequent loss of the W6/32 epitope. Furthermore the B*4402 complexes failed to improve their thermostability over the pulse chase period. In contrast, B*4402 complexes loaded in the presence of tapasin became progressively more thermostable over time, having undergone tapasin dependent and time dependent peptide optimisation. The 4°C stable complexes were all 37°C stable at 30 minutes and there was no loss of complexes over the chase period. Furthermore the B*4402 complexes continued to optimise their peptide load, enabling the entire cohort of complexes to be 50°C stable at 120 minutes. These observations are consistent with the idea that MHC class I complexes that are unstable at physiological temperatures are prone to degradation and the degradation and optimisation are competing processes. This optimisation may involve the exchange of suboptimal peptides for those with a higher affinity or trimming of peptides of suboptimal length.

The failure of B*4402 to optimise its peptide repertoire may be interpreted as an absolute failure to initially load peptides. I think that this is unlikely as Figure 4.3.1.2 has previously shown that the introduction of ICP47 further impairs the thermostability, suggesting that the B*4402 complexes in 220 are peptide occupied. This inability of B*4402 peptide optimisation in the absence of tapasin may illuminate the primary role of tapasin as a facilitator of MHC class I complex peptide optimisation or as having an indirect effect upon such processes. One such indirect effect would be the stabilisation of TAP and hence improved

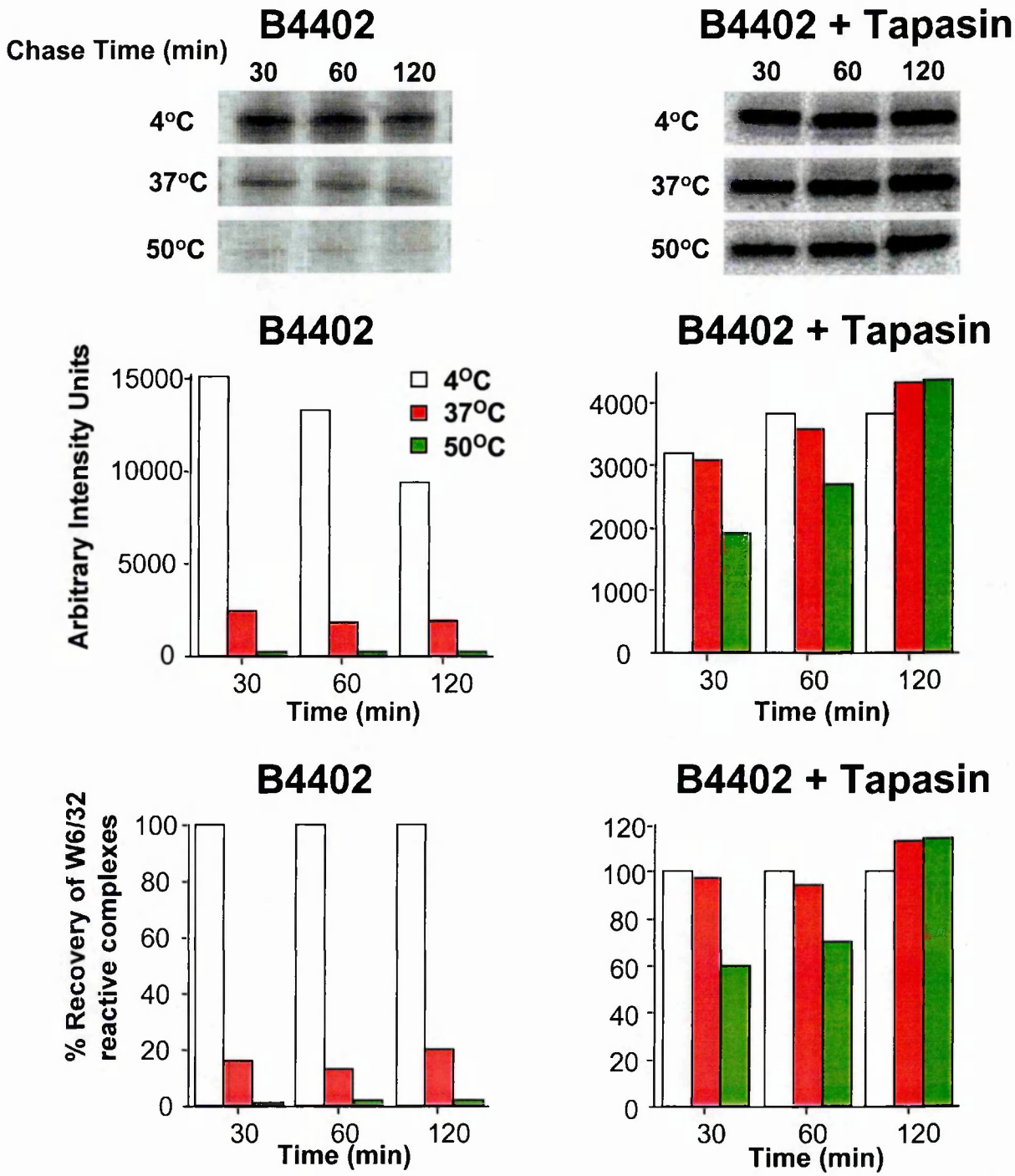


Figure 4.5.2.1 – Pulse chase Thermostability assay of B*4402 +/- Tapasin.

B*4402 complexes were evaluated in the presence an absence of tapasin over a 2 hour time period. The 4°C stable material diminished over the 2 hour period in the absence of tapasin whilst in the presence of tapasin an on-going improvement in the thermostability of the complexes could be seen.

peptide supply. This is unlikely to be the entire reason as B lymphoblastoid cell lines constitutively over-express TAP (Rowe et al., 1995)and soluble tapasin, which does not effect TAP levels, restores cell surface B*4402 expression (see Results 4.6). Furthermore, when 220.B*4402 cells were pre-treated with IFN-γ to further maximise TAP expression and peptide translocation prior to pulse chase thermostability analysis there was no change in the profile of B*4402 (Figure 4.5.2.2). When exogenous peptide is added to cell cultures to bypass TAP delivery there is no improvement in the thermostability of the B*4402 complexes. Only when peptide is added at the time of cell lysis is B*4402 able to load peptide independent of tapasin (Figure 4.5.2.2). Comparison with the 220.B*4402.Tapasin.ICP47 cell line shows that there is an improved thermostability profile even in the absence of TAP peptides when tapasin is present, possibly reflecting the improved usage and optimisation of any existing ER or TAP independent peptide sources. Finally, a tapasin mutant with an N terminal deletion that permits TAP stabilisation but does not allow a tapasin MHC class I complex interaction to form was introduced into 220.B*4402. Figure 4.5.2.3 shows that there was no improvement in the peptide repertoire of B*4402 under these conditions. Therefore tapasin appears to be required for the optimisation of the peptide repertoire of B*4402. The on-going improvement in the thermostability of tapasin even when it has left the ER is intriguing. Perhaps this relates to peptide trimming, tapasin independent peptide exchange or further conformational changes that make the complex more thermostable.

(i)

	220.B*4402							
	No IFN-γ				IFN-γ			
	30 mins		120 mins		30 mins		120 mins	
	Au	%	Au	%	Au	%	Au	%
4	7269	100	5176	100 (↓71)	9323	100	5972	100 (↓64)
37	1376	19	935	18	1793	19	1190	20
50	173	2	178	3	293	3	219	4

(ii)

	220.B*4402			220.B4402.Tapasin. ICP47	
	-	+	Lysis	-	+
	%	%	%	%	%
4	100	100	100	100	100
37	17	19	60	44	42

Figure 4.5.2.2 – Pulse chase Thermostability assay of 220. B*4402 +/- IFNγ and following exogenous peptide loading

B*4402 complexes show no improvement in thermostability following IFNγ usage (i). B*4402 does not load exogenously pulsed SEIDTVAKY peptide but does acquire peptide following cell lysis (ii). B*4402 is more thermostable in the presence of tapasin even when the peptide supply is limiting (220.B*4402.Tapasin.ICP47)

	220.B*4402.NDel44Tapasin					
	30 mins		60 mins		120 mins	
	Au	%	Au	%	Au	%
4	1743	**100**	910	**100 (↓52)**	531	**100 (↓30)**
37	295	**17**	265	**29**	238	**45**
50	55	**3**	61	**7**	98	**18**

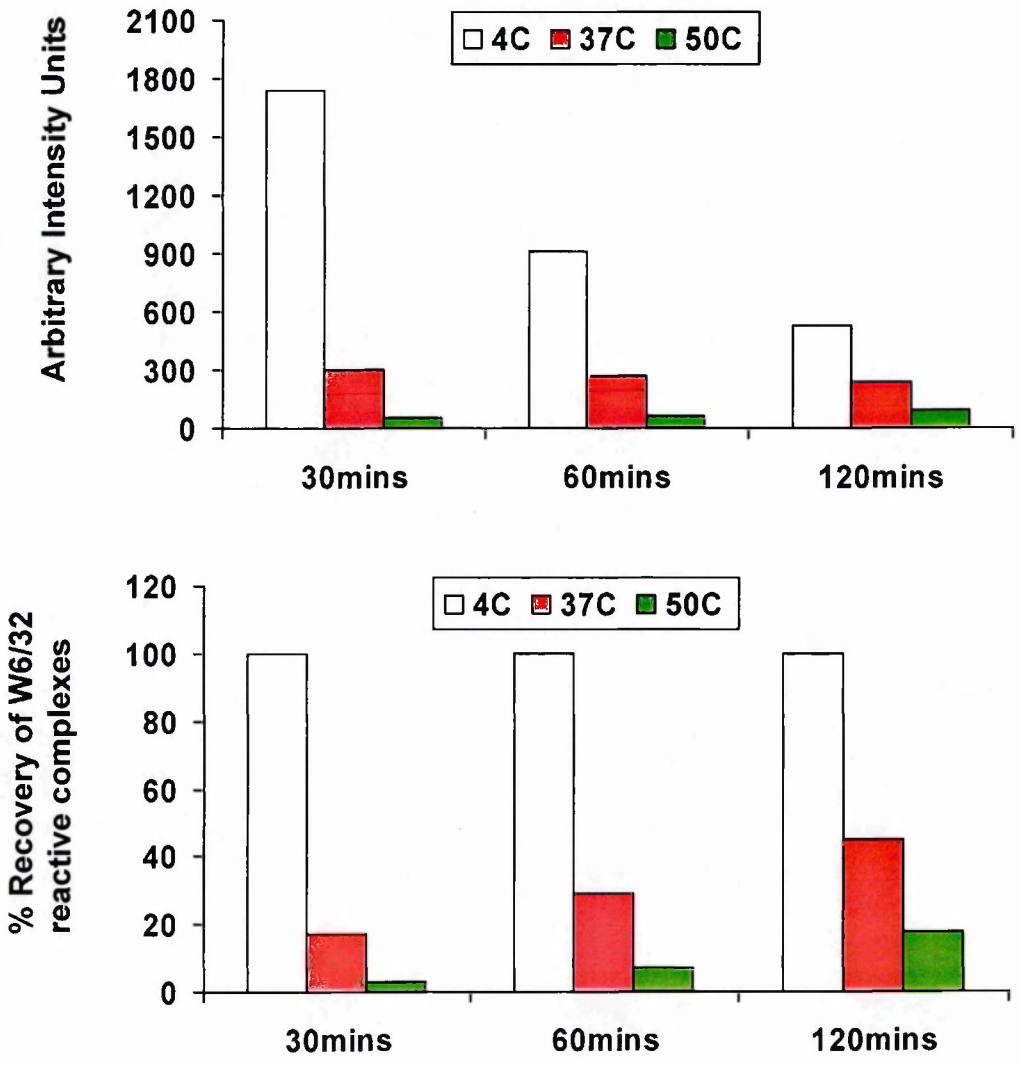


Figure 4.5.2.3 – Pulse chase Thermostability assay of 220. B*4402. NDel44 Tapasin.

Failure to optimise B*4402 in the presence of a N terminal mutant of tapasin which restores TAP function but does not permit a PLC to form.

4.5.3 TAPASIN INDEPENDENT TIME DEPENDENT OPTIMISATION OF THE B*2705 PEPTIDE REPERTOIRE

Figure 4.5.3.1 shows the comparable data set on the B*2705 allele. In first considering the differences between the presence and absence of tapasin 3 points can be made. Firstly in the absence of tapasin there is a progressive loss of 4°C material over time which is not seen in the presence of tapasin. This is similar but less marked to that seen for B*4402. Secondly the introduction of tapasin greatly enhances the thermostability of B*2705 complexes, with 37°C stability occurring early and continued 50°C optimisation seen throughout the chase period. Thirdly time dependent optimisation is seen for B*2705 in the absence of tapasin. The 50°C stable complexes increase over the duration of the pulse chase as does their ratio compared to the 37°C stable complexes. The ratio of 50°C: 37°C stability changes from 0.24 at 30 minutes to 0.61 at 120 minutes (Table 4.5.3.1). This is a better guide in this cell line as some of the 4°C material is lost from the assay, whilst the 37°C material is stable. This ratio change is also seen for the tapasin competent cells with ratios of 0.61 at 30 minutes rising to 0.84 at 120 minutes. Thus the capacity of B*2705 complexes to traffic to the cell surface in the absence of tapasin, despite the fact that the newly assembled complexes are unstable, may relate to this property of tapasin independent time dependent optimisation. Again it could be argued that these complexes are not optimised but are originally empty and then filled with optimal peptide. This is unlikely for at least 2 reasons. Firstly, the ratio of 50°C: 37°C

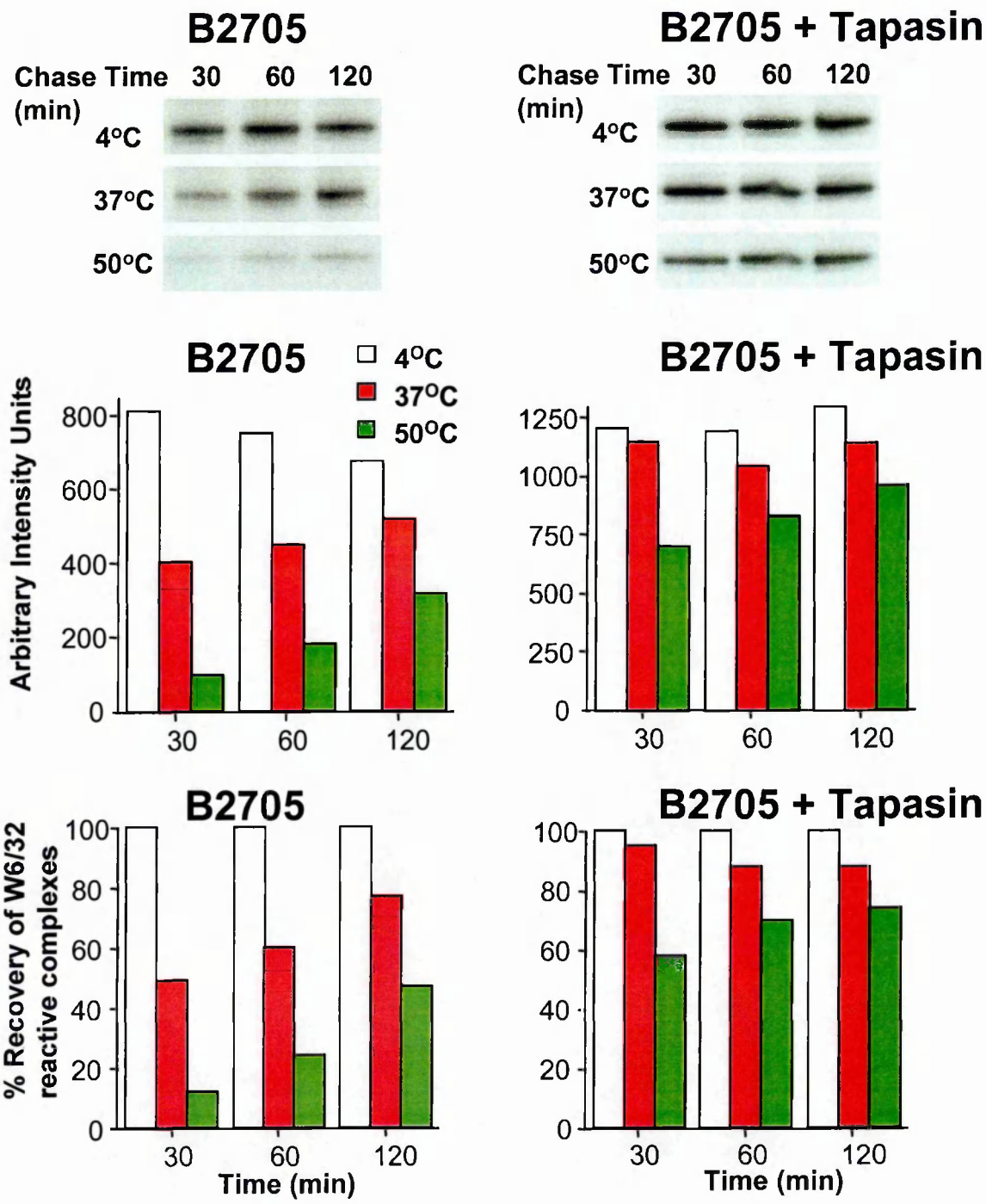


Figure 4.5.3.1 – Pulse chase Thermostability assay of B*2705 +/- Tapasin.

B*2705 complexes were evaluated in the presence an absence of tapasin over a 2 hour time period. The 4°C stable material diminished over the 2 hour period in the absence of tapasin whilst in the presence of tapasin an on-going improvement in the thermostability of the complexes could be seen.

	50°C:37°C stability ratio		
	30 mins (AU)	60 mins (AU)	120 mins (AU)
220.B*2705	**0.24** (95:461)	**0.41** (182:447)	**0.61** (316:517)
220.B*2705.Tapasin	**0.61** (696:1145)	**0.80** (831:1044)	**0.84** (958:1140

Table 4.5.3.1 – Comparative stability of B*2705 complexes over time +/- Tapasin

The 37°C material is stable during the pulse chase period, reflecting the peptide cargo bound by the B*2705 complexes. The 50°C stable material continues to increase during the pulse chase period. The ratio between the 2 thermostable cohorts changes over time with an increased ratio of the 50°C stable cohort to the 37°C cohort. This suggests that there is either new recruitment into the 50°C cohort or that the 37°C cohort is becoming 50°C stable.

stable complexes increase over time suggesting that previously peptide occupied complexes are further refining their peptide repertoire as discussed previously for B*4402. Secondly when ICP47 is introduced there is a deterioration in the stability of B*2705 complexes within pulse label thermostability studies (Figure 4.3.1.2) and a blockade of time dependent optimisation within pulse chase studies (Figure 4.5.3.2). Furthermore when such experiments are undertaken in the presence of BFA, optimisation continues, further supporting the role of ER peptides in the time dependent optimisation of B*2705. Therefore B*2705 is similar to B*4402 in requiring tapasin for maximal optimisation but differs in that it is able to undergo tapasin independent, time dependent optimisation that is reliant on a supply of TAP translocated peptides.

4.5.4 A COMPARISON OF THE ROLE OF TAPASIN FOR THE OPTIMISATION OF B*4402 AND B*2705

Figure 4.5.4.1 compares the pulse chase thermostability profiles of B*4402 and B*2705 loaded in the presence of tapasin. Both alleles maintain the 4°C material throughout the chase period and attain 37°C stability by 30 minutes. For B*2705 all complexes are stable to 37°C at the 30 minute time point. This is in contrast to that achieved for B*2705 in the absence of tapasin at any time point during the 2 hour chase period. Therefore tapasin appears to offer B*2705 advantages in the both the rate and extent of optimal peptide loading. As both alleles are able to acquire a 50°C stability it is possible to compare the thermostabilities between

(i)

	220.B*2705				220.B*2705 + BFA			
	30 mins		120 mins		30 mins		120 mins	
	AU	%	AU	%	AU	%	AU	%
4	2710	**100**	1799	**100 (↓66)**	2879	**100**	2080	**100 (↓72)**
37	1048	**38**	1343	**75**	1161	**40**	1475	**71**

(ii)

	220.B*2705				220.B*2705.ICP47			
	30 mins		120 mins		30 mins		120 mins	
	AU	%	AU	%	AU	%	AU	%
4	3865	**100**	3074	**100 (↓80)**	4227	**100**	3165	**100 (↓75)**
37	1315	**34**	1897	**62**	944	**22**	1125	**36**

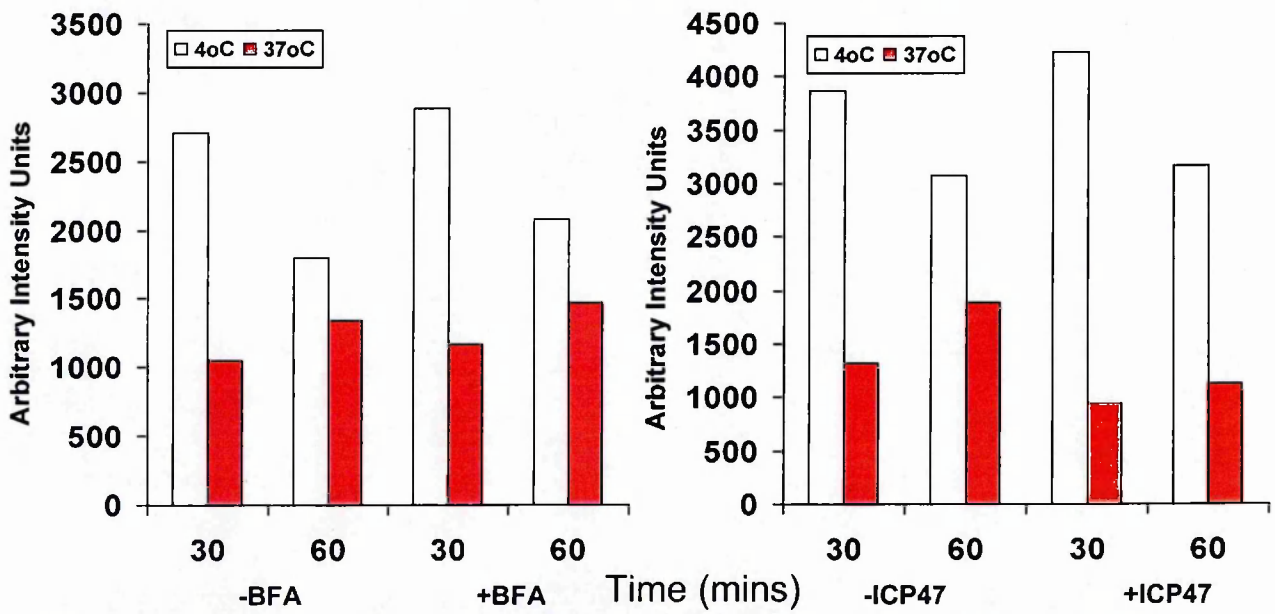


Figure 4.5.3.2 – Pulse chase Thermostability assay of B*2705 +/- ICP47 and +/- Brefeldin A (BFA)

(i) B*2705 complexes were evaluated in the presence an absence of BFA over a 2 hour time period. No differences were seen in the optimisation profiles.
(ii) B*2705 complexes were evaluated in the presence and absence of ICP47. The presence of ICP47 decreased the time dependent increase in thermostability

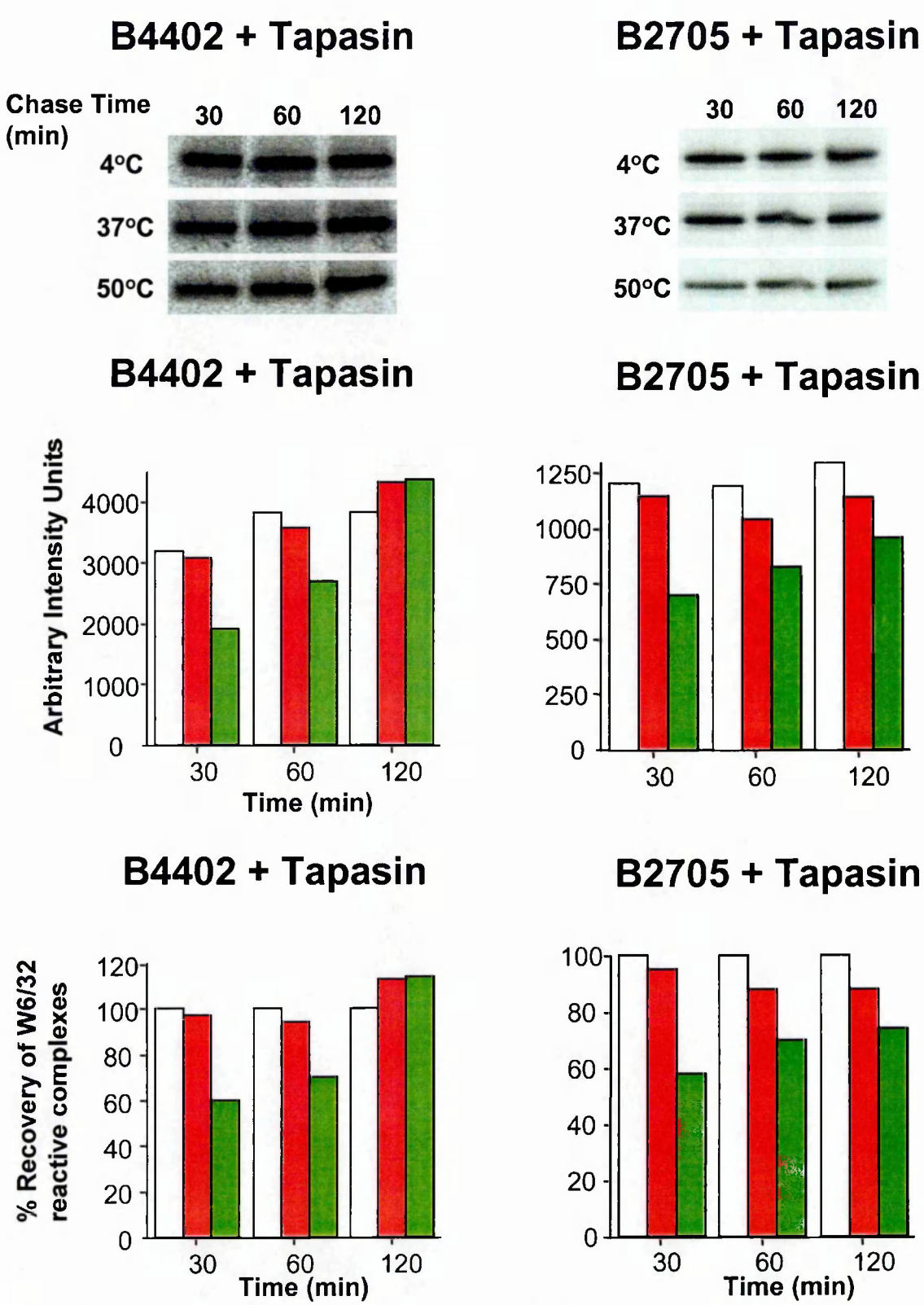


Figure 4.5.4.1 – Pulse chase Thermostability assay of B*2705 and B*4402 in the presence of Tapasin.

B*4402 and B*2705 were evaluated in the presence of tapasin. Both alleles showed an improved stability of the 4°C material throughout the 2 hour time period. Also the attainment of 37°C stability was seen for both alleles at the earliest time point.

different alleles. B*4402 can be seen to achieve maximal 50°C thermostability at 120 minutes whilst B*2705 only achieved 74%. Therefore the extent of tapasin facilitated optimisation is greater for B*4402. To further examine influence of tapasin upon the rate of optimisation, a 2 minute label short pulse chase thermostability assay was undertaken. Figure 4.5.4.2 shows that at 6 minutes 80% of the newly synthesised B*4402 MHC class I complexes are 37°C stable. As further newly synthesised complexes emerge between 6-12 and 12-18 minutes their 37°C stability is also maintained. There is also evidence of the early stages of further optimisation as the 50°C:37°C ratio begins to improve between the 6 and 18 minute time points. Figure 4.5.4.3 shows the results for B*2705, again emphasising the speed of optimal peptide acquisition with 74% of complexes 37°C stable at 6 minutes rising to 87% at 18 minutes. Therefore tapasin can be seen to both enhance the rate of optimisation and the extent of peptide loading. A catalyst will normally speed up the rate at which equilibrium is attained but will not alter the position of the equilibrium. Tapasin may therefore act to enhance the rate of optimisation in an analogous manner to a catalyst but it's effects upon the extent of optimisation is a secondary consequence. This may relate to the rate enhancement (i.e. competition of degradation) or another function of tapasin (i.e. direct stabilising effect upon MHC class I complexes). These possibilities are dealt with further in the discussion. This optimisation of the peptide repertoire is a defining property of tapasin that is seen most readily in the context of these thermostability assays.

	220.B*4402.Tapasin					
	6 mins		12 mins		18 mins	
	AU	%	AU	%	AU	%
4	949	100	1515	100	2110	100
37	760	80	1387	92	1733	82
50	418	44	905	60	1200	58
50:37 stability ratio	**0.55** (418:760)		**0.65** (905:1387)		**0.69** (1200:1733)	

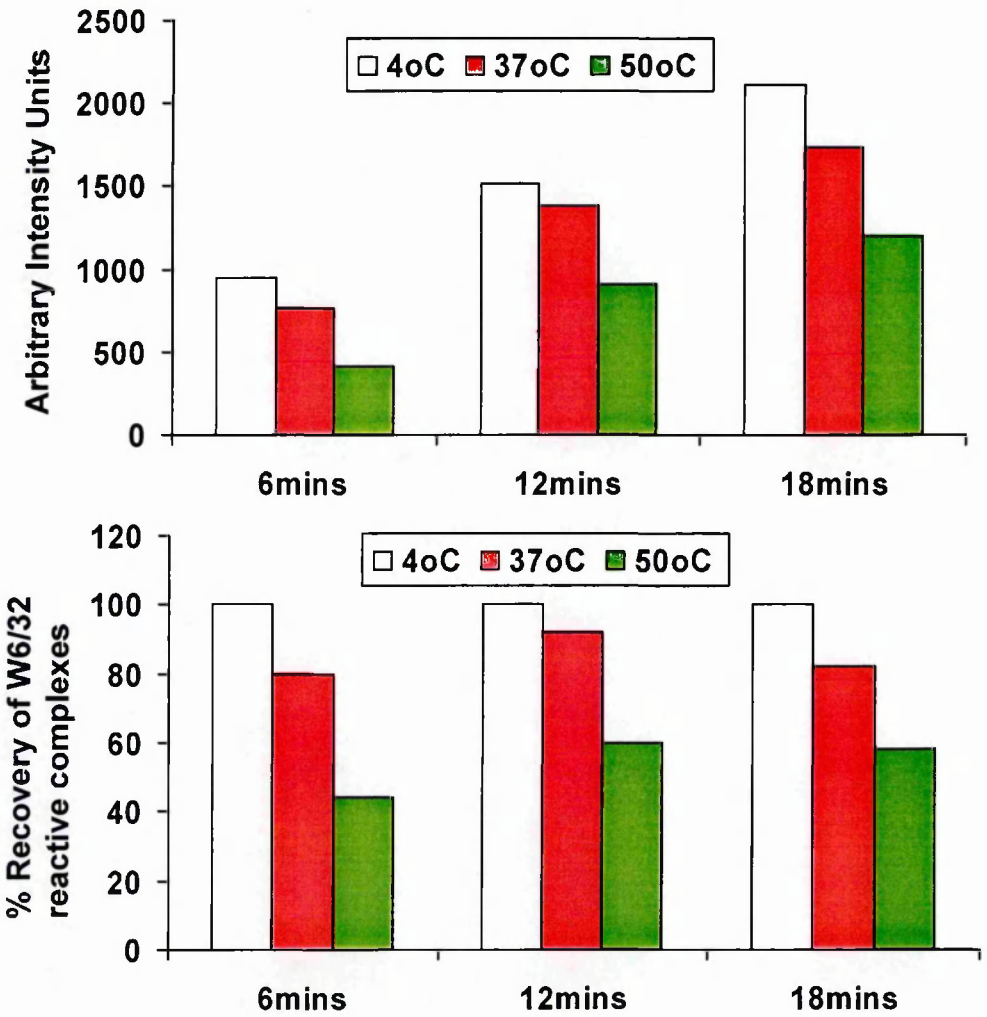


Figure 4.5.4.2 – Short pulse chase Thermostability assay of B*4402 in the presence of Tapasin.

B*4402 complexes were followed following a 2 minute label at 6,12 and 18 minutes. Early attainment of 37°C stability is seen at each time point.

	220.B*2705.Tapasin					
	6 mins		12 mins		18 mins	
	AU	%	AU	%	AU	%
4	1090	100	1634	100	1974	100
37	811	74	1417	87	1719	87
50	627	57	968	59	1370	69
50:37 stability ratio	0.77 (627:811)		0.68 (968:1417)		0.80 (1370:1719)	

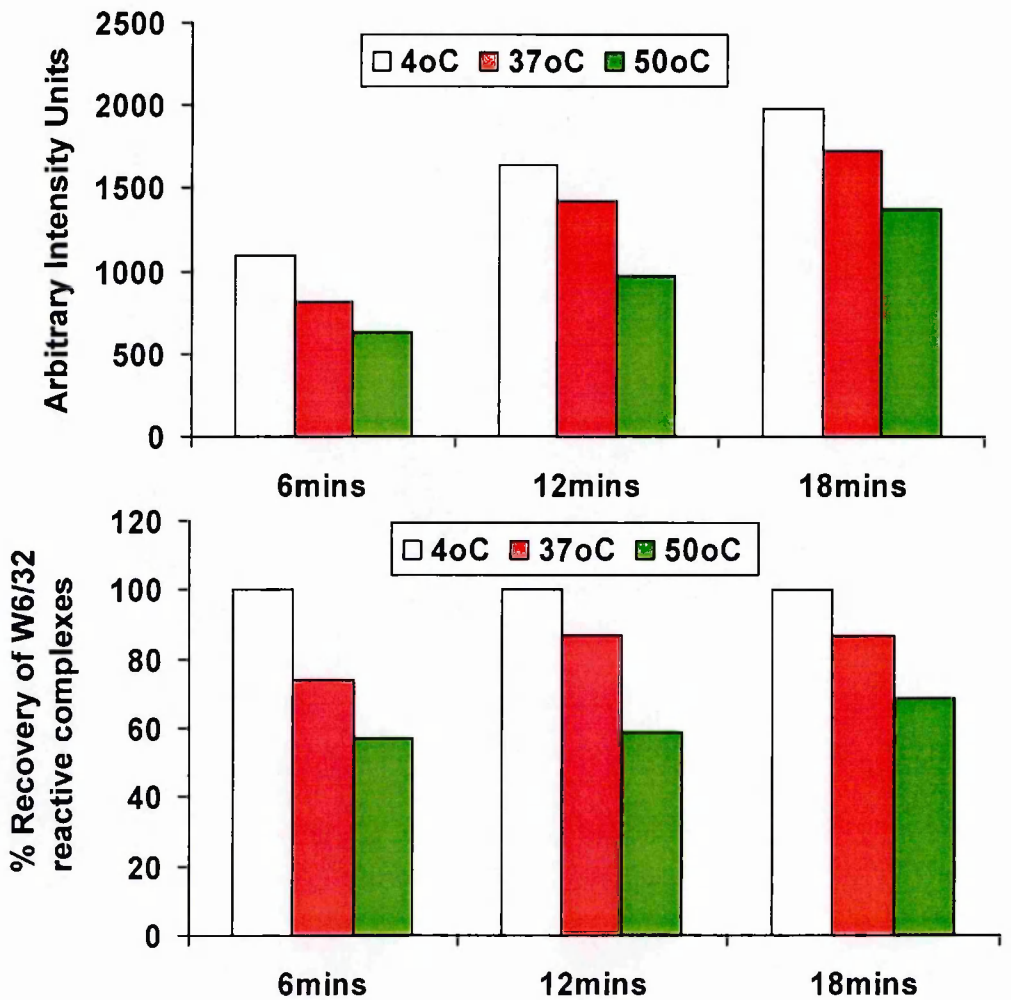


Figure 4.5.4.3 – Short pulse chase Thermostability assay of B*2705 in the presence of Tapasin.

B*2705 complexes were followed, after a 2 minute label, at 6,12 and 18 minutes. Early attainment of 37°C stability is seen at each time point. The extent of 50°C stability is similar to that seen with B*4402 at these early time points.

4.5.5 INVESTIGATION OF THE EFFECT OF TAPASIN ON B*4402 AND B*2705 TIME DEPENDENT OPTIMISATION WITH REGARD TO THE ENDOGLYCOSIDASE SENSITIVE AND RESISTANT COHORTS

A further analysis of the role of tapasin in optimising the peptide repertoires of B*4402 and B*2705 was undertaken through studying the endoglycosidase H sensitive and resistant MHC complex cohorts. Table 4.5.5.1 shows the results of the pulse chase characteristics of B*4402 in the presence of tapasin. In the absence of tapasin no trafficking of B*4402 from the ER could be identified. Additional pulse experiments undertaken at 26°C still failed to show an endo R cohort of molecules (data not shown). In the presence of tapasin, half maximal trafficking is seen at approximately 30 minutes. The trafficking times of the 37°C and 50°C cohorts were slightly faster than the total cohort of molecules at each time point. Figure 4.5.5.2 examines the thermostabilities of the endo S and R fractions at each time point. Notably, all of the endo R complexes are 37°C stable, whilst most of the endo S pool was 37°C stable at each time point. Furthermore at the end of the chase period 90% of the endo S material is 37°C stable with 55% being 50°C stable, compared to 81% and 37% at the 30 minute time point. Therefore, as no material is lost in the presence of tapasin, the material still within the ER has undergone optimisation over this time period. The endo R cohort is all 37°C stable at 120 mins with 83% achieving 50°C stability. It therefore appears that even the complexes that do not egress the ER are still

220.B*4402.Tapasin Endo H +/- Differential pulse chase kinetics for thermostable complexes .				
	15 MINS	**30 MINS**	**60 MINS**	**120 MINS**
4^{0}C				
ENDO H R	**24%**	**50%**	**69%**	**87%**
ENDO H S	76%	50%	31%	13%
37^{0}C				
ENDO H R	**33%**	**58%**	**78%**	**91%**
ENDO H S	67%	42%	22%	9%
50^{0}C				
ENDO H R	**31%**	**60%**	**81%**	**91%**
ENDO H S	69%	40%	19%	9%

Figure 4.5.5.1 – Pulse chase characteristics of different thermostable B*4402 cohorts in the presence of tapasin.

Typical pulse chase calculations enumerate the fraction of total complexes that have achieved endo R status at each time point. This is typically undertaken with all the complexes kept at 4°C. B*4402 does not traffic in the absence of tapasin. In the presence of tapasin endo R material is seen as early as 15 minutes with half maximal trafficking at 30 minutes. The more thermostable cohorts have slightly faster trafficking rates when compared to the total 4°C stable material.

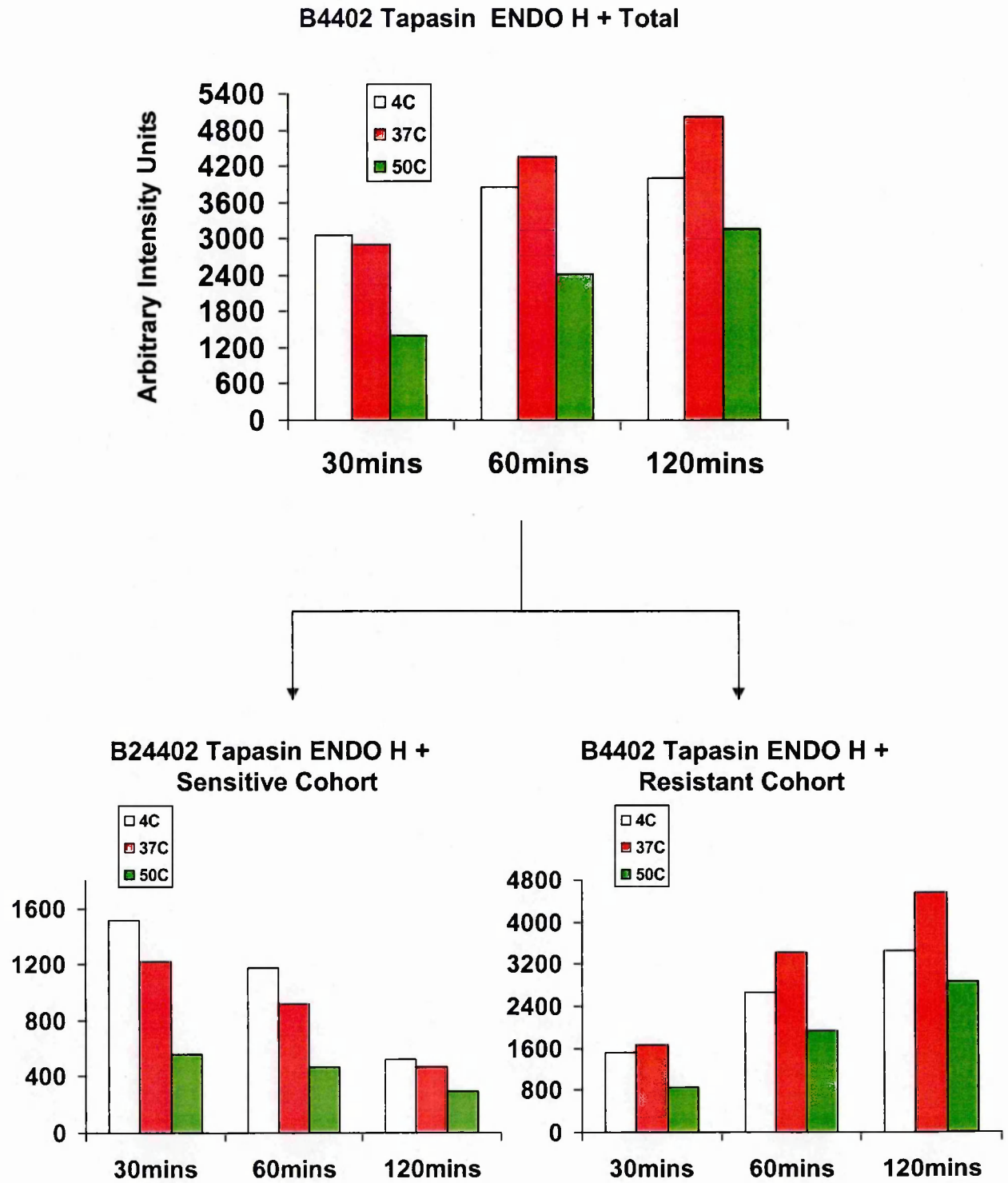


Figure 4.5.5.2 – Cohort analysis of the pulse chase Thermostability assay of B*4402.Tapasin +/- Endo H digestion.

The endo H + experiment allows for the representation of 2 cohorts of class I molecules. The endo S cohort are distinct and may enter the endo R cohort (or ERAD) whilst the endo R cohort may undergo further 'autonomous' change (post ER) and be modified by trafficking endo S complexes between time points.

able to undertake peptide optimisation whilst those endo R complexes can improve their profiles still further. The details of how and where this further optimisation occurs and the degree to which the recently exported complexes contribute to this observation may now be examined through such an experimental design. One such set up will be to 'trap' the endo R cohort of B*4402 by culturing cells in deoxymannojirimycin (dMJ) which inhibits ERM I and II. This could be timed to act when approximately 50% of the complexes would be expected to have become endo H resistant. .The action of dMJ will then prevent endo H cleavage of the emerging B*4402 complexes, allowing then to transverse the trans-golgi normally but with an endo S phenotype. The B*4402 complexes that have previously left the ER can then be analysed in the absence of a contribution from newly emerging complexes. Figure 4.5.5.3 shows the detailed thermostability breakdown of the endo S and R complexes. It is interesting that the 50°C: 37°C stability ratio increased in the endo R pool in excess of that seen in the endo S pool. This may reflect the continuing improvement in endo S 37°C complexes which continually enter the endo R pool. However these figures are at best semi quantitative and in deriving these figures a modification of the endo R figures has been made. This modification was to use the 4°C pool as the denominator and assume all of these complexes are 37°C stable (as the 37°C complex arbitrary unitage was greater than that of the 4°C pool). Taking all this into account it would still appear that there is disproportionate increase in the 50°C stable pool within the endo R fraction. The molecular mechanism of this continual change is not clear but could include

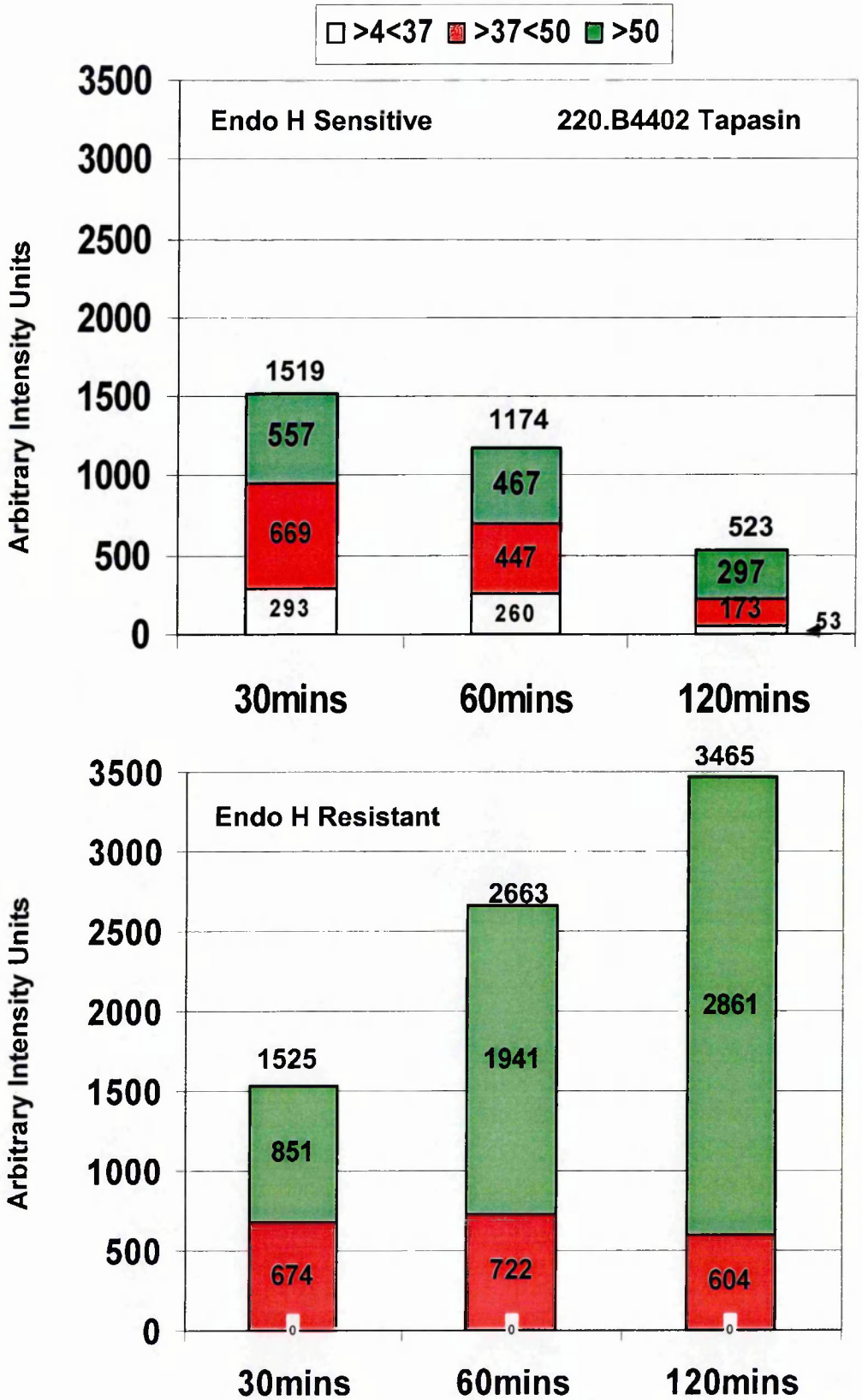


Figure 4.5.5.3 – Evaluation of the origin of endo R thermostable B*4402 complexes in the presence of tapasin

An sub-analysis of three different thermostable cohorts of B*4402 is seen within either the endo S material (ER resident) or the endo R material (post medial golgi).

peptide trimming and exchange (as mentioned previously) or possibly change in the tertiary structure of the MHC class I complex. Such changes may include sialylation, glycan modification or a change in the conformation of the MHC class I complex. Such changes have been described for MHC class II peptide complexes analysed as recombinant molecules in vitro (Sadegh-Nasseri et al., 1994). Examining the endo R material in the absence of a continuing influx from the endo S pool (i.e. dMJ experiment) would also enable a clearer look at this matter. Finally, an examination of a single chain peptide MHC class I complex would be fascinating in this regard. Would such a complex that has its peptide cargo 'fixed' show an improvement over time? Furthermore would complexes with a restricted peptide repertoire (i.e. HLA-E) also show an improvement in thermostability over time when peptide exchange/trimming might be expected to play less of a role during their maturation?

B*2705 can be analysed in an analogous manner, both in the presence and absence of tapasin. Figure 4.5.5.4 shows the 4°C pulse chase characteristics of B*2705 do not differ markedly in the presence and absence of tapasin, with only a slight increase in trafficking seen in the absence of tapasin. B*2705 is seen to optimise its peptide cargo within this endo H + thermostability set up (Figure 4.5.5.5). The loss of 4°C material is again seen between 60 and 120 minutes. Whereas there is loss from 4°C material between the 30 and 60 minute time points in the endo S fraction, a comparable increase in the endo R material is seen. The loss of the 4°C material between 60 and 120 minutes can be further scrutinised in Figure 4.5.5.6 where the composite thermostabilities are plotted.

220.B*2705. Endo H +/- Differential pulse chase kinetics for thermostable complexes .				
	15 MINS	30 MINS	60 MINS	120 MINS
4°C				
ENDO H R	40%	55%	70%	75%
ENDO H S	60%	45%	30%	25%
37°C				
ENDO H R	54%	69%	78%	88%
ENDO H S	46%	31%	22%	12%
50°C				
ENDO H R	58%	70%	84%	90%
ENDO H S	42%	30%	16%	10%

220.B*2705.Tapasin Endo H +/- Differential pulse chase kinetics for thermostable complexes .				
	15 MINS	30 MINS	60 MINS	120 MINS
4°C				
ENDO H R	35%	40%	55%	66%
ENDO H S	65%	60%	45%	34%
37°C				
ENDO H R	38%	43%	55%	68%
ENDO H S	62%	57%	45%	32%
50°C				
ENDO H R	36%	42%	56%	64%
ENDO H S	64%	58%	44%	36%

Figure 4.5.5.4 – Pulse chase characteristics of different thermostable B*2705 cohorts in the absence and presence of tapasin.

B*2705 complexes can egress the ER in the absence of tapasin. At the earlier time -points the complexes loaded in the presence of tapasin appear to traffic slower than those loaded in the absence of tapasin at all thermostabilities.

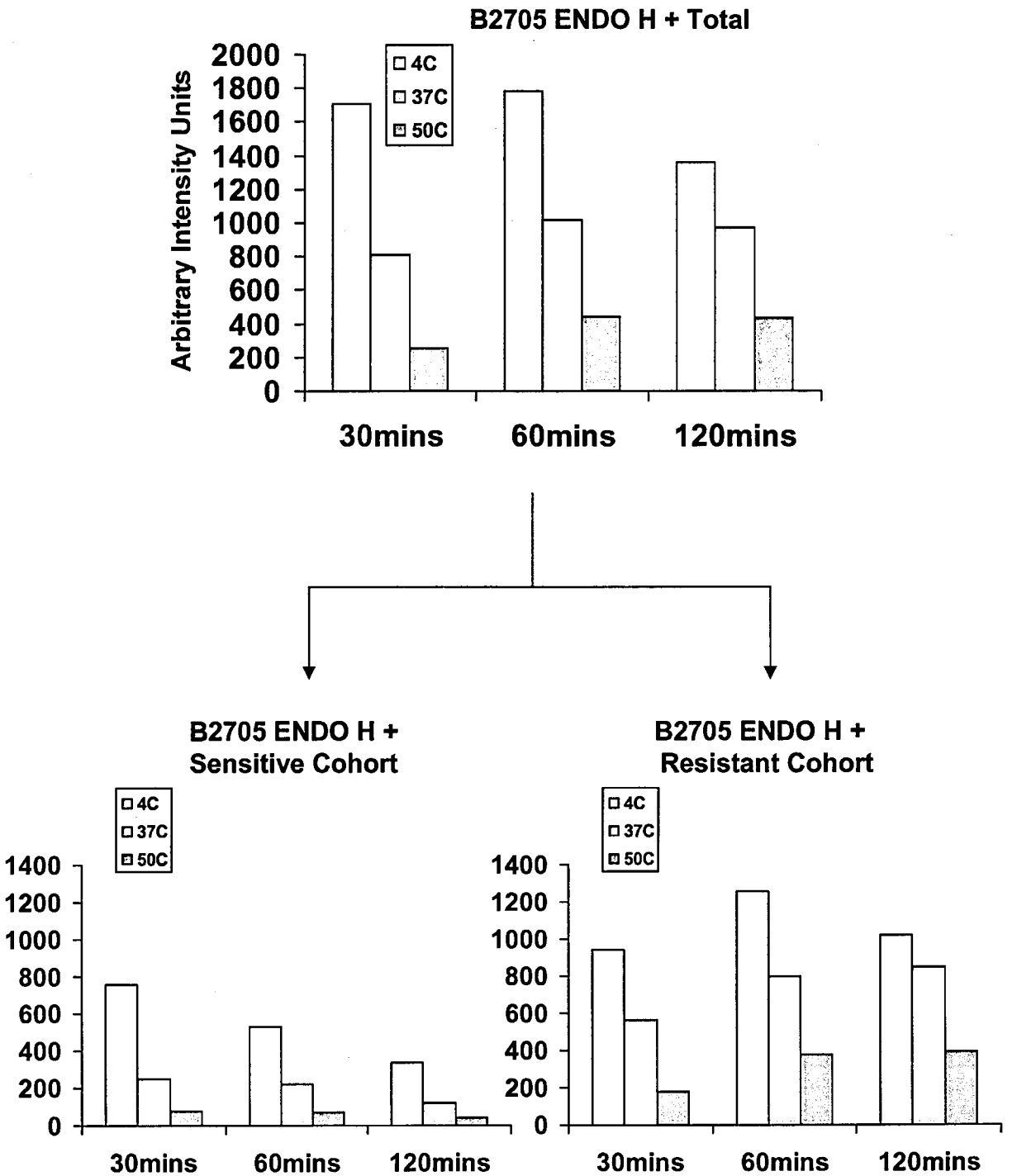


Figure 4.5.5.5 – Cohort analysis of the pulse chase Thermostability assay of B*2705 +/- Endo H digestion.

There appears to be a 429 AU loss between 60 and 120 minutes (1787-1358) with 232 AU lost from the 4°C stable endo R pool and 197 AU lost from the endo S pool. This loss from the 60 minute endo S pool is not accounted for in the 120 minute endo R pool and has thus probably been degraded. The other losses from the endo R pool may represent complexes that have dissociated en route to the cell surface or at the cell surface. This loss may represent 4°C stable but 37°C unstable complexes. Despite this complex loss from the endo R material it is clear that the endo R complexes are more thermostable then the endo S complexes but neither are ever entirely 37°C stable. In contrast to the B*4402 data (Figure 4.5.5.3), the endo R material is maximally optimised at 60 minutes and only appears to loose '>4°C <37°C' (WHITE) material after this time. When B*2705 acquires peptide in the presence of tapasin a different picture is seen. Figure 4.5.5.7 shows that both the endo S and R cohorts are comparably stable as early as 15 minutes post pulse label. During the chase period there is a further improvement in the 50°C stable material. This change is principally seen in the endo R material whilst the endo S material remains qualitatively similar. Figure 4.5.5.8 details the temperature stability make up of the different cohorts and shows a steadily increasing '>50°C stable material cohort' between 30 and 60 minutes. This ongoing increase in the stability of the endo R material is less marked than that of B*4402 and may entirely reflect the increased thermostability of the imported endo S material between 60 and 120 minutes. Why peptide loaded thermostable B*2705 complexes remain as endo S material within the ER is not clear. However, these latter ER complexes are more thermostable than

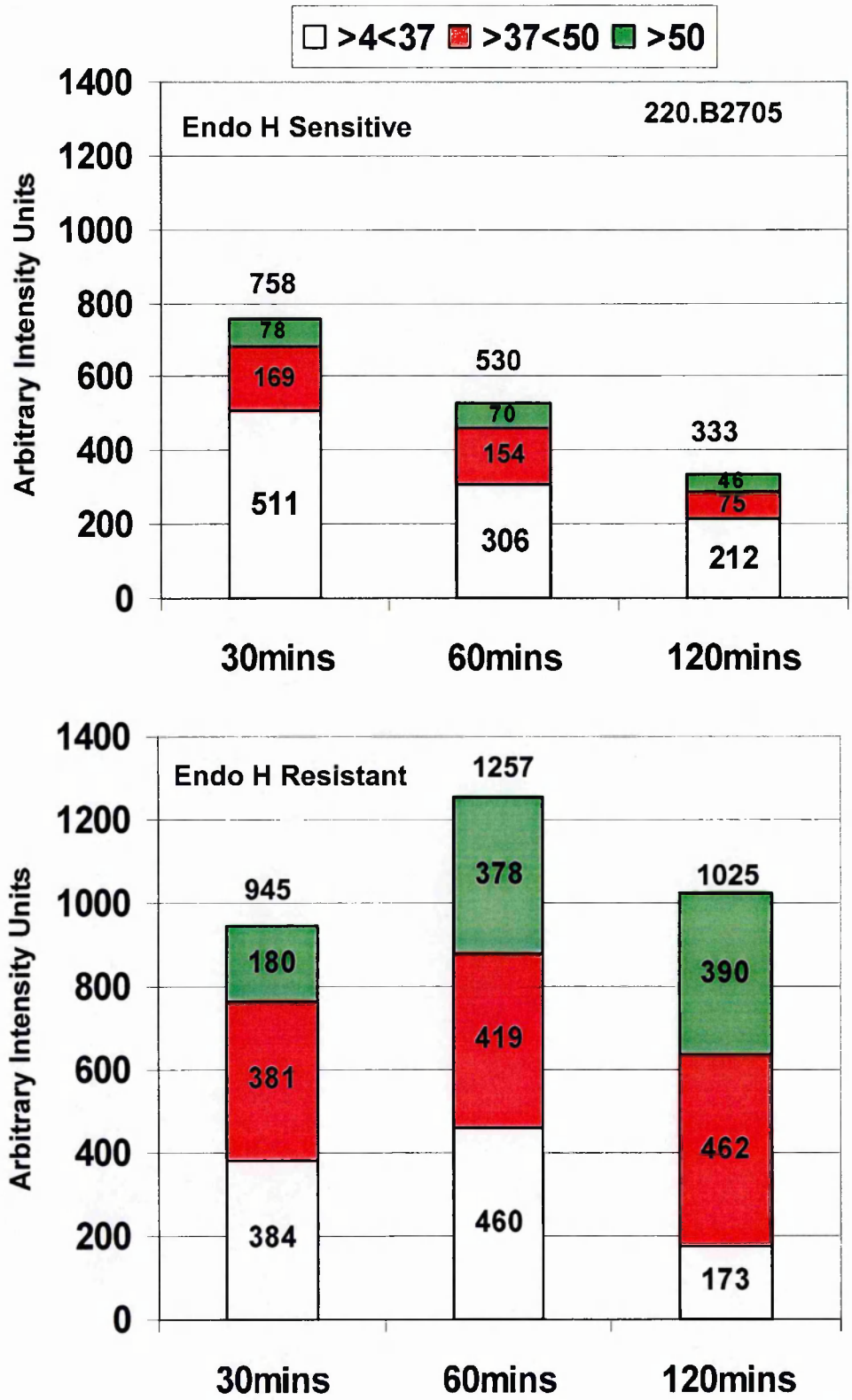


Figure 4.5.5.6 – Evaluation of the origin of endo R thermostable B*2705 complexes in the absence of tapasin.

An sub-analysis of three different thermostable cohorts of B*2705 is seen within either the endo S material or the endo R material.

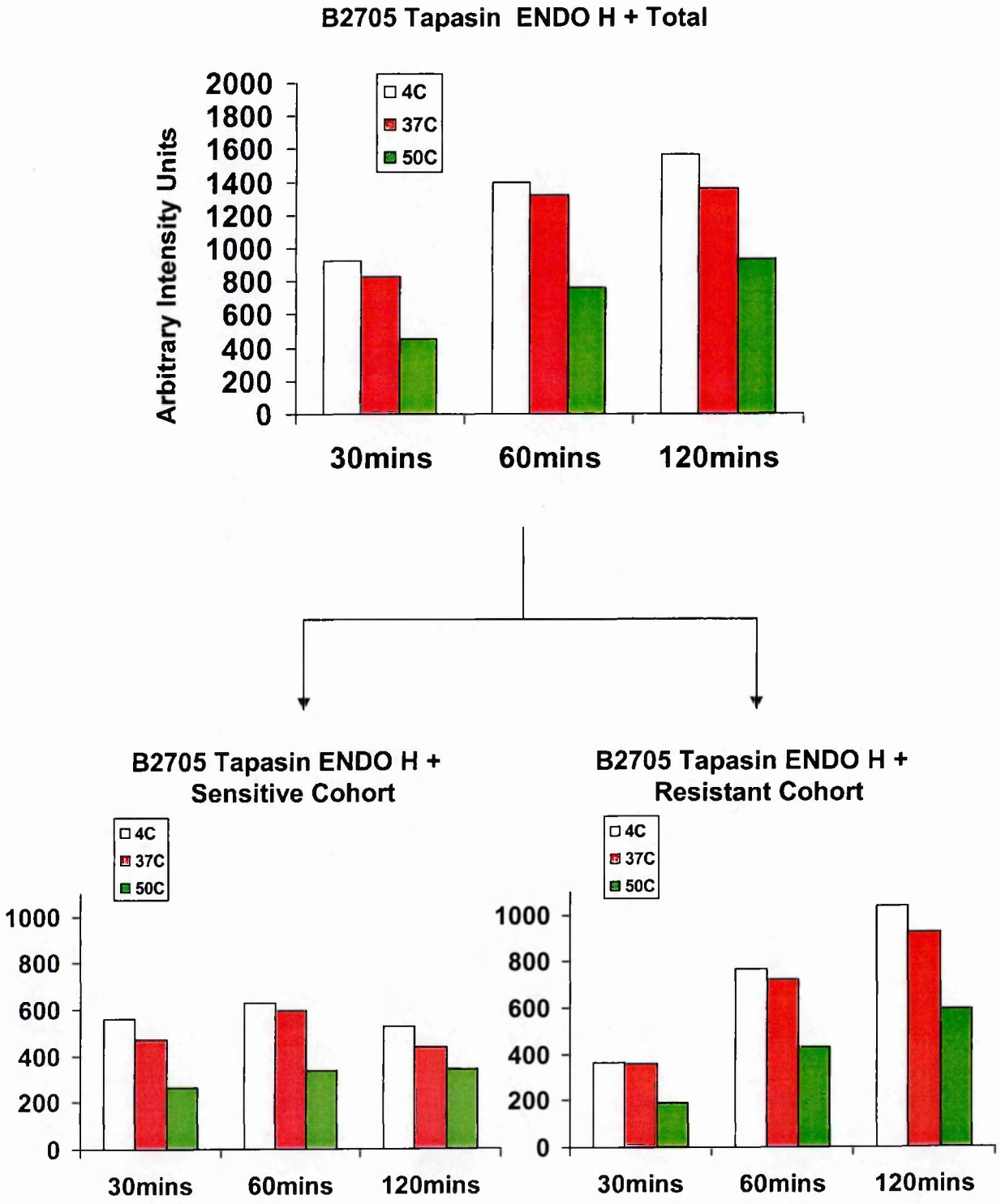


Figure 4.5.5.7 – Cohort analysis of the pulse chase Thermostability assay of B*2705.Tapasin +/- Endo H digestion.

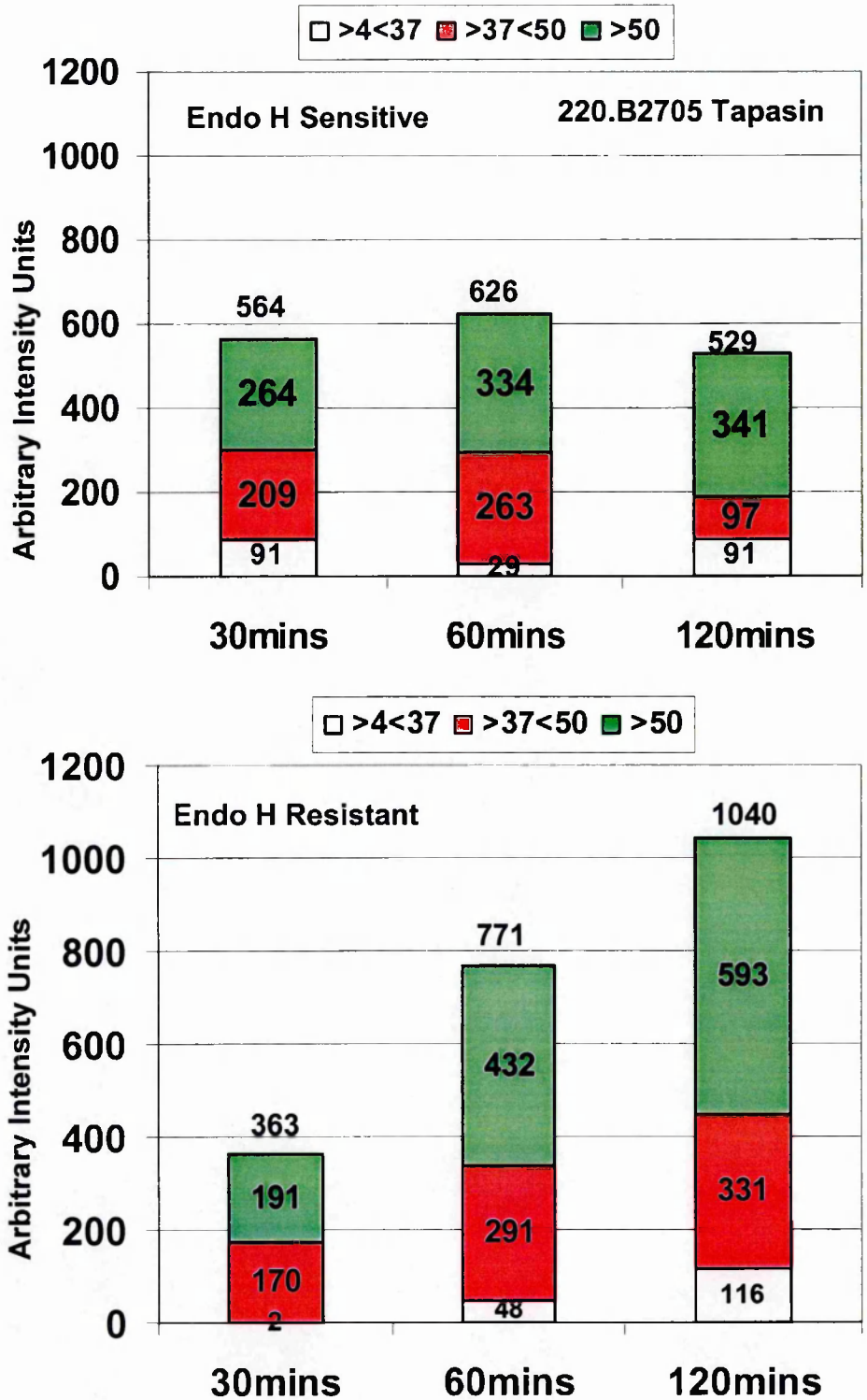


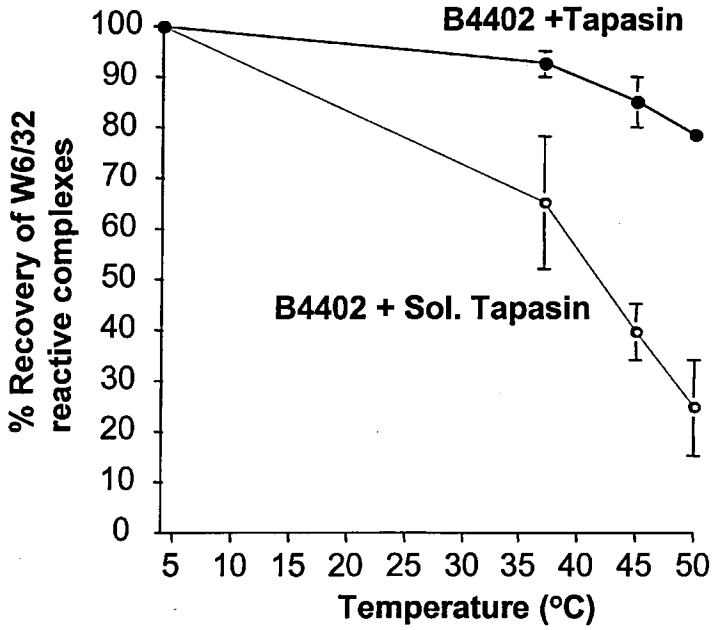
Figure 4.5.5.8 – Evaluation of the origin of endo R thermostable B*2705 complexes in the presence of tapasin.

An sub-analysis of three different thermostable cohorts of B*2705 is seen within either the endo S material or the endo R material.

those seen at 30 minutes and as there is no loss of B*2705 material between 30 and 120 minutes this would further support the notion of ER mediated optimisation of peptide occupied complexes.

4.6 ASSESSMENT OF THE MHC CLASS I COMPLEX-TAP INTERACTION FOR PEPTIDE OPTIMISATION.

To further examine the role of tapasin in facilitating peptide optimisation I choose to investigate that PLC bridging function of tapasin. It had been previously shown that soluble tapasin, lacking its transmembrane and cytoplasmic domains, could restore the surface expression and antigen presentation of HLA-B8, despite the failure of soluble tapasin to restore the interaction between HLA-B8 and TAP (Lehner et al., 1998). I explored this 'TAP uncoupling' soluble tapasin construct in the context of B*4402 and B*2705 peptide loading. Soluble tapasin fully restored the cell surface expression of both B*4402 and B*2705. Figure 4.6.1 shows the effect of soluble tapasin upon B*4402 in the pulse label thermostability assay. Whilst there is an obvious improvement over the previously shown 220.B*4402 thermostability plots, consistent with restored cell surface expression, there is a clear difference from the loading seen in the presence of full length tapasin. As soluble tapasin does not influence the TAP levels this improved peptide loading reflects the utilisation of a peptide pool that was present in 220.B*4402 cells but could not be utilised in the absence of a B*4402 tapasin interaction. The majority of complexes are stable at 37°C but



B*4402 Tapasin	Duplicate 1	Duplicate 2	Mean
	% of 4^{0}C	% of 4^{0}C	
4	100	100	**100**
37	90	95	**93**
45	90	80	**85**
50	77	80	**79**

B*4402 Soluble Tapasin	Duplicate 1	Duplicate 2	Mean
	% of 4^{0}C	% of 4^{0}C	
4	100	100	**100**
37	78	52	**65**
45	45	34	**40**
50	34	15	**25**

Figure 4.6.1 – Thermostability of B*4402 +/- Soluble Tapasin.

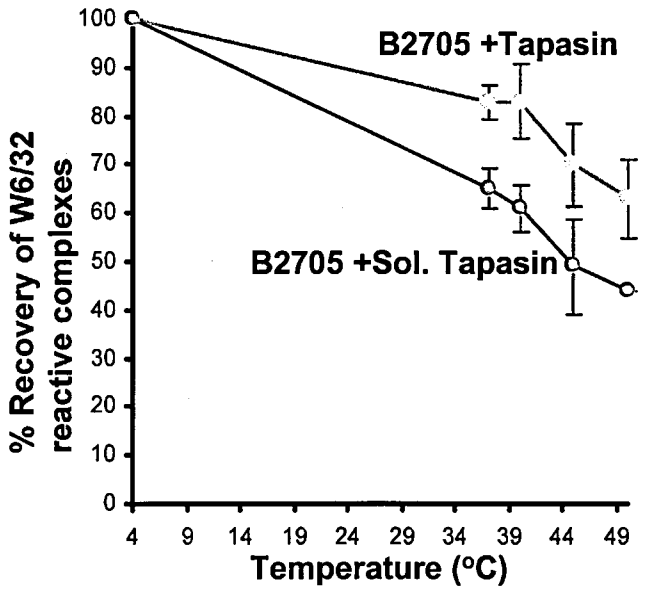
Duplicate experiments of B*4402 +/- soluble tapasin were quantified and plotted.

they differ markedly from B*4402 loaded in the presence of full length tapasin at all higher temperatures. These differences may relate to different pools of peptides, poorly trimmed peptides or a combination of such factors.

When B*2705 is examined in an identical manner a similar set of results are observed. Figure 4.6.2 illustrates an improved thermostability compared to that seen in the absence of tapasin but an inferior profile compared to full length tapasin.

The B*4402 and B*2705 complexes were then assessed over a 2 hour pulse chase period. Figure 4.6.3 shows the results for B*4402. When compared with the optimisation profiles in the presence of full length tapasin (Figure 4.5.2.1) an inferior initial and end point optimisation is seen. There is no loss of complexes over the pulse chase, suggesting that all complexes are loaded but that this repertoire is suboptimal. This is not due to faster trafficking of B*4402 in the presence of soluble tapasin as pulse chase analysis shows identical trafficking rates (data not shown). At 120 minutes the 37°C stable cohort represents 89% and 50°C stable cohort 56% of all 4°C stable material. The ratio of 50°C: 37°C material increased over time from 0.45 at 30 minutes to 0.63 at 120 minute. This is consistent with soluble tapasin dependent optimisation. It is also notable that the profile is similar to full length tapasin with an important improvement in 50°C stability occurring between 60 and 120 minutes (31%>56%).

Figure 4.6.4 shows the results for the B*2705 allele. Again the introduction of tapasin abrogates the loss of 4°C material but the optimisation profile is decreased when compared to full length tapasin (Figure 4.5.3.1). To further

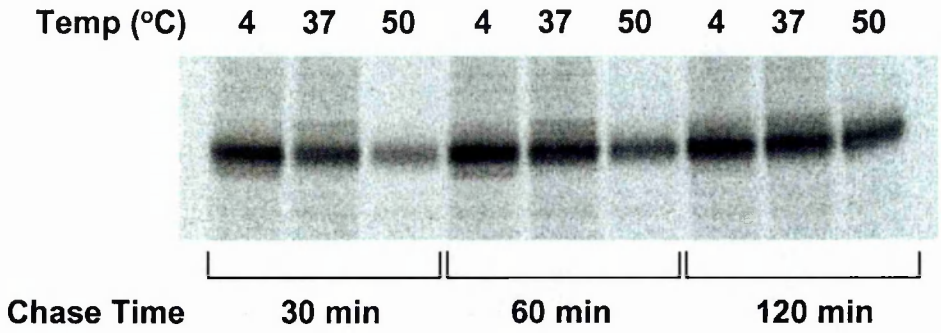


B*2705 Tapasin	Triplicate 1	Triplicate 2	Triplicate 3	Mean
	% of 4°C	% of 4°C	% of 4°C	
4	100	100	100	100
37	79	84	86	83
45	78	70	61	69
50	55	71	62	63

B*2705 Soluble Tapasin	Duplicate 1	Duplicate 2	Mean
	% of 4°C	% of 4°C	
4	100	100	100
37	62	68	65
45	42	56	49
50	44	44	44

Figure 4.6.2 – Thermostability of B*2705 +/- Soluble Tapasin.

Replicate experiments of B*2705 +/- soluble tapasin were quantified and plotted.



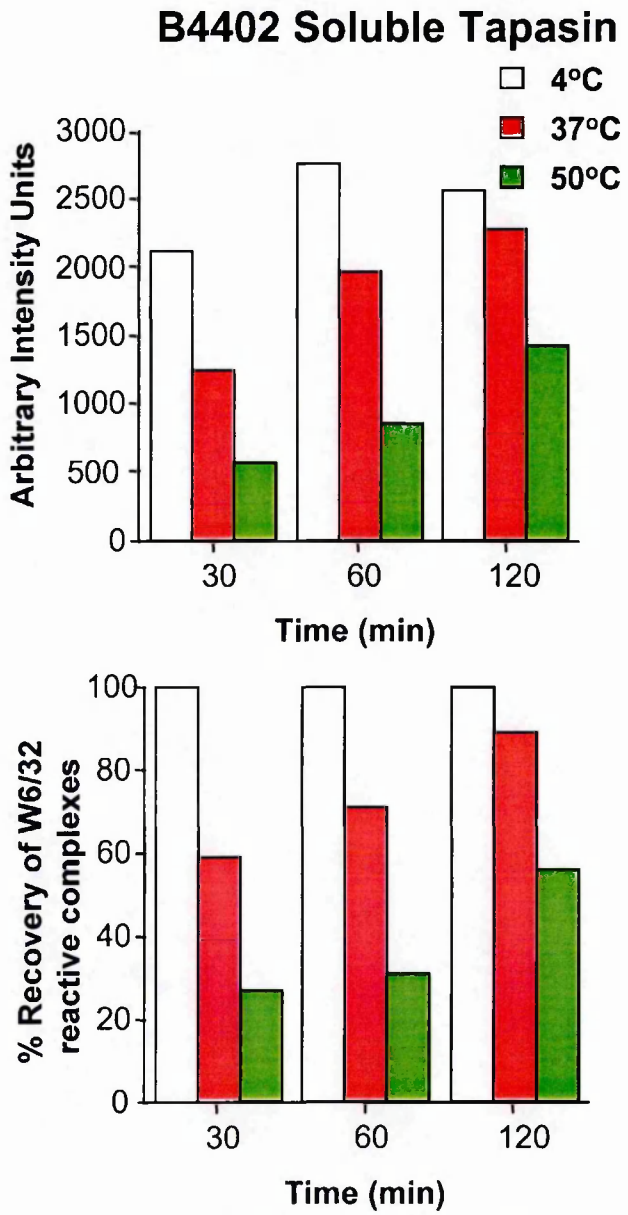


Figure 4.6.3 – Pulse chase Thermostability assay of B*4402 complexes in the presence of soluble tapasin.

B*4402 complexes were evaluated in the presence an absence of soluble tapasin over a 2 hour time period.

	220.B*2705.Soluble Tapasin					
	30 mins		60 mins		120 mins	
	Au	%	Au	%	Au	%
4	704	100	810	100	628	100
37	399	57	397	49	375	60
50	289	41	248	31	268	43

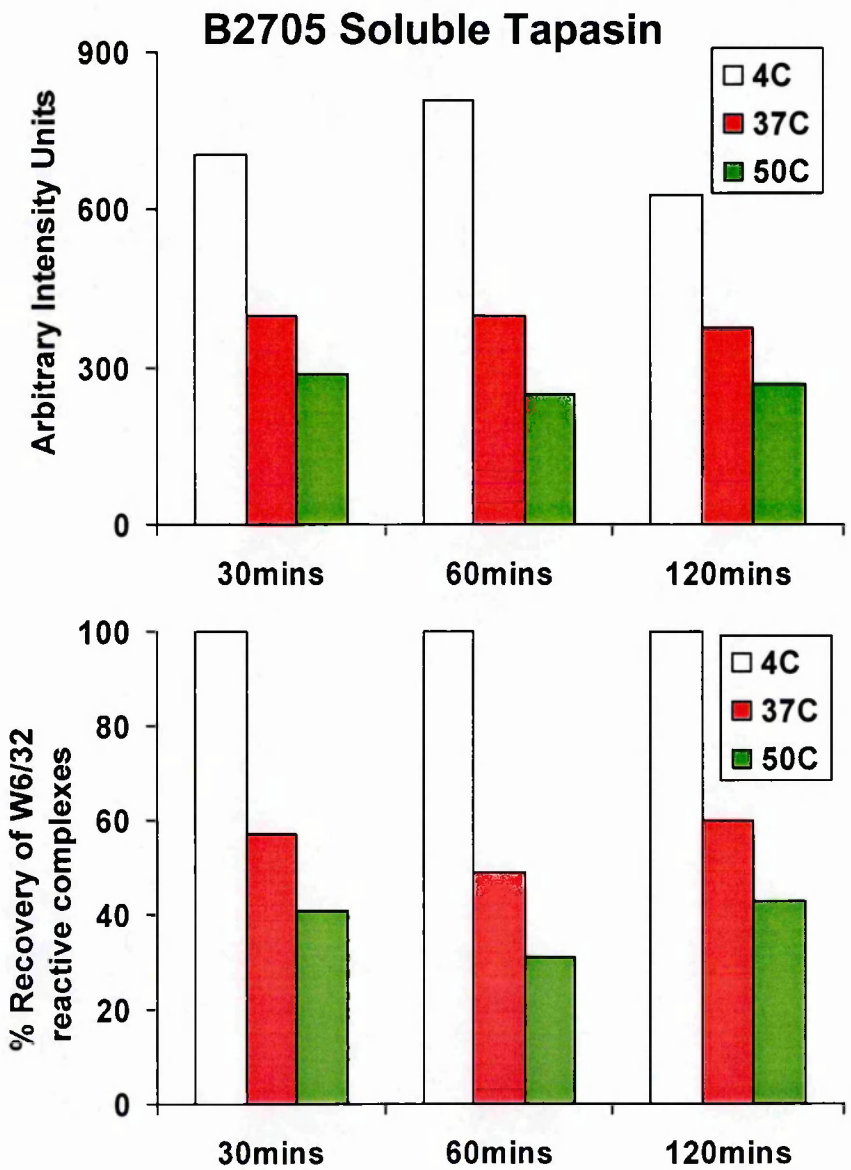


Figure 4.6.4 – Pulse chase Thermostability assay of B*2705 complexes in the presence of soluble tapasin.

B*2705 complexes were evaluated in the presence an absence of soluble tapasin over a 2 hour time period.

compare the stability of the complexes I undertook a BFA decay experiment of cell surface material across the B*4402 and B*2705 cell lines. Figure 4.6.5 shows that the differences detected by the thermostability assay are reflected in the decay characteristics of the cell surface material. B*4402 and B*2705 are both more stable at the cell surface when loaded in the presence of full length tapasin. Also, B*4402 is always seen to be more stable that B*2705 when compared across full length or soluble tapasin cell lines.

It appears that soluble tapasin can only partially substitute for full length tapasin in promoting peptide loading. This may due to a number of direct and indirect effects. A failure to fully recruit the complete PLC apparatus to the soluble MHC class I complex has been postulated (Tan et al., 2002). Additionally, the failure to optimally trim peptides or encounter the maximum number of peptides available when the TAP interaction is disrupted, are other possibilities. However the restoration of peptide loading for B*4402 indicates that the interaction between lumenal tapasin and the MHC class I complex is essential for the utilisation of the pre-existing peptide pool. This initial and/or ongoing interaction permits a repertoire of peptide selection and time dependent optimisation that is sufficient to permit egress of B*4402 complexes to the cell surface.

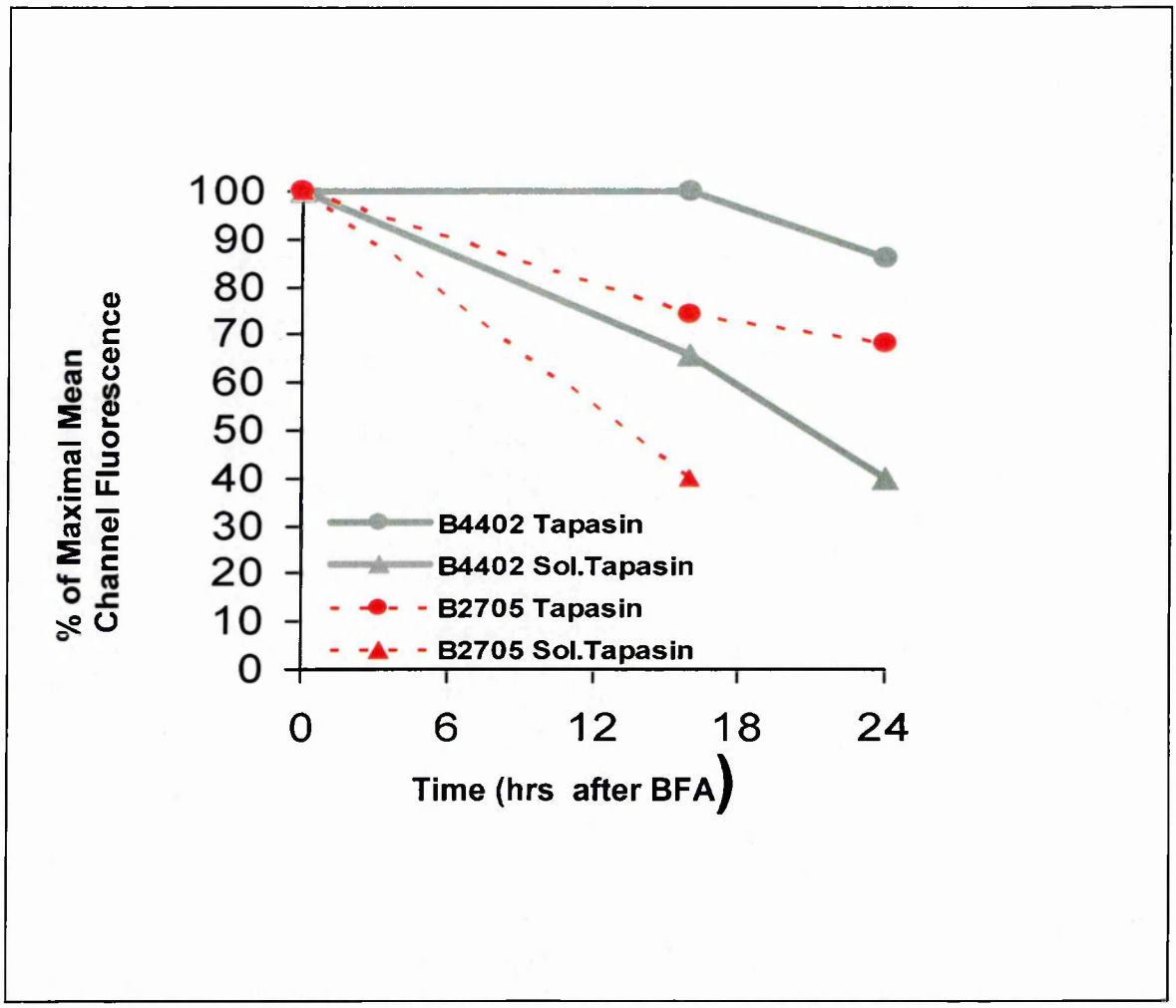


Figure 4.6.5 – Comparative BFA decay of B*4402 and B*2705 in the presence of full length or soluble tapasin.

Cell surface complexes were assessed at 18 and 24 hours following the addition of BFA to block the egress of newly formed complexes to the cell surface. The MFI of the complexes at the cell surface at each time point is presented as a percentage of that seen at the start of the experiment.

4.7 A SINGLE B*4402 POINT MUTATION CONVERTS THE COMPLEX TO A TAPASIN INDEPENDENT ALLELE (B*4405)

The distinct differences in the behaviour of the B*4402 and B*2705 were further investigated through a comparison of their primary structures and integration of the literature on chaperone associations. Although position 116 is not one of the 20 amino acid differences between B*4402 and B*2705, it's contribution to both peptide selection and PLC interaction prompted us to asses an aspartic acid (D) to tyrosine (Y) change at this position in B*4402. This mutated HC is identical to the naturally occurring B*4405 allele. Remarkably, this single change was able to permit the expression of B*4405 at the cell surface in the absence of tapasin. Further more the behaviour of this allele was akin to B*2705 with comparable cell surface expression in tapasin competent and incompetent environments (Figure 4.7.1).

Following this observation we performed pulse label thermostability experiments and compared them to those with B*4402 (Figure 4.7.2). The profiles were very similar to those seen with B*2705, showing poor stability of B*4405 complexes in the absence of tapasin and enhanced stability in its presence. The stability of B*4405 was always greater than that of B*4402 in the absence of tapasin, whilst the opposite was true in the presence of tapasin.

Figure 4.7.3 compares the pulse chase thermostability characteristics of all 4 cell lines. The B*4402 thermostability plots are similar to those previously seen with early maximal 37°C stability and ongoing 50°C enhancement. The B*4405

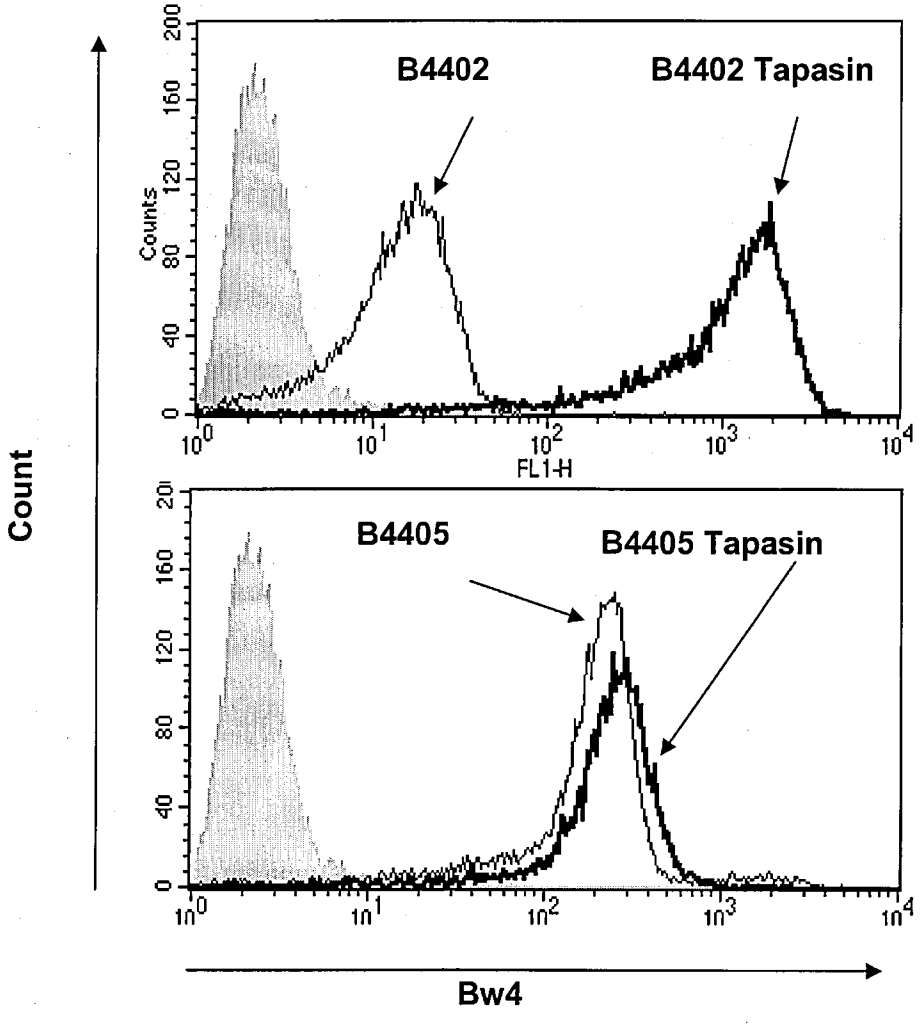


Figure 4.7.1 – FACS profiles of B*4402 and B*4405 in the presence and absence of tapasin

Representative FACS plots of the steady state levels of B*4402 and B*4405 in the presence and absence of tapasin

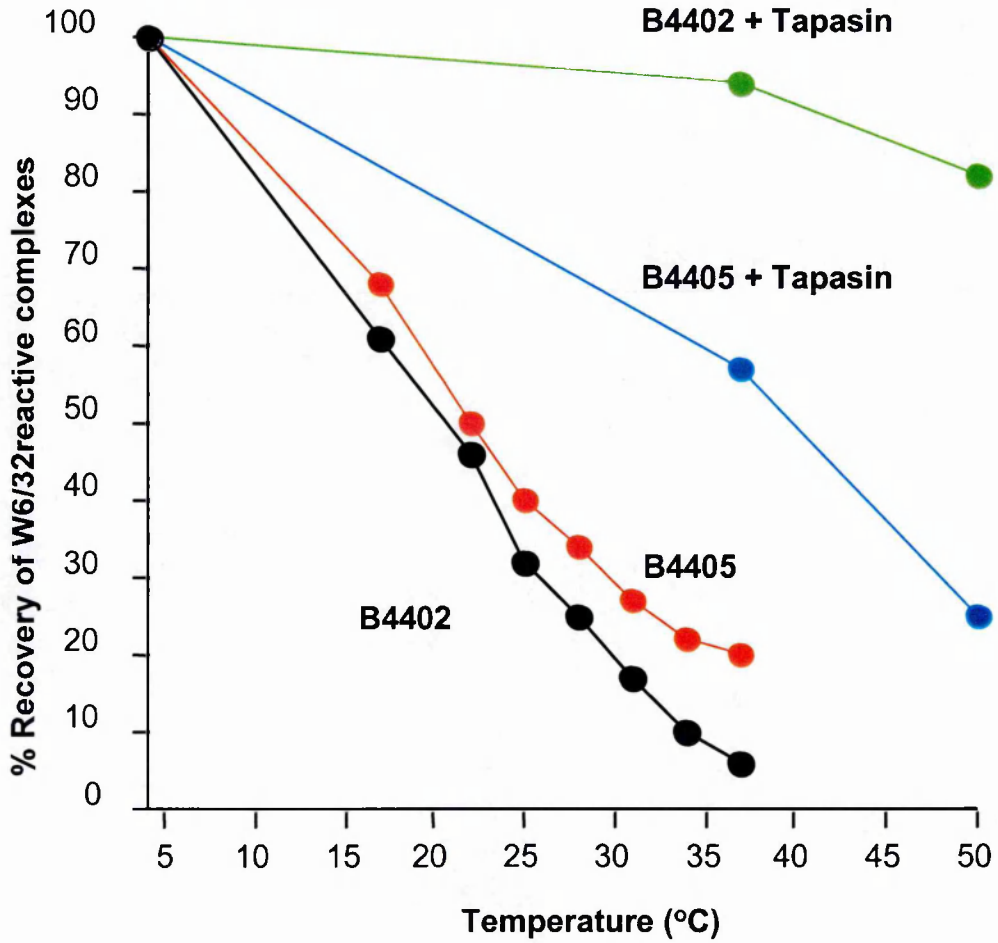


Figure 4.7.2 – Thermostability of B*4402 and B*4405 +/- Tapasin.

Comparative thermostability experiments were undertaken on the cDNA transfectants of B*4402 and B*4405 in the presence and absence of tapasin. B*4405 shows a poor thermostability in the absence of tapasin which improves upon tapasin transfection. B*4405 is more thermostable than B*4402 at all temperatures.

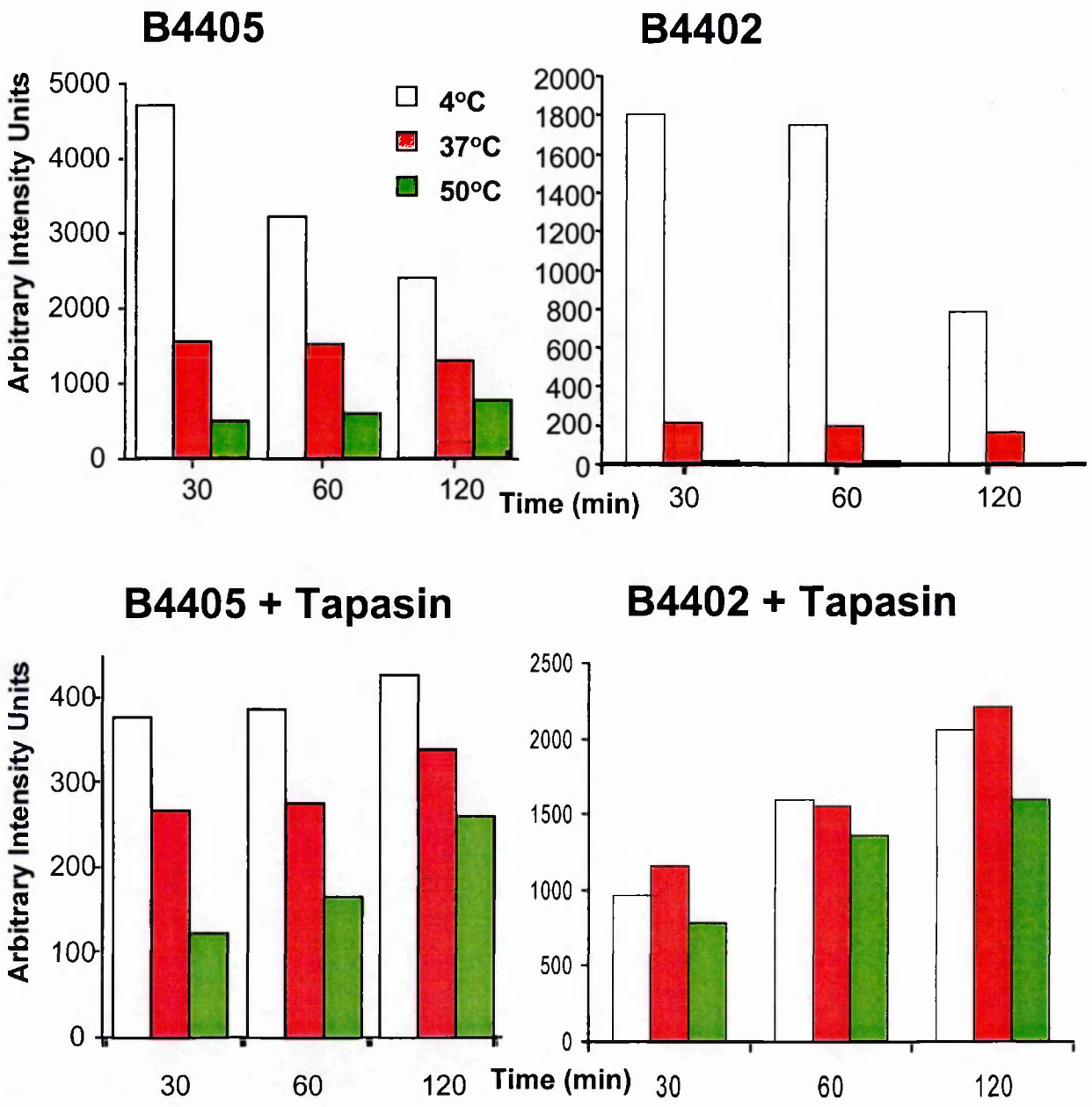


Figure 4.7.3 – Pulse chase Thermostability of B*4402 and B*4405 +/- Tapasin

Pulse chase thermostability assays wee undertaken on B*4402 and B*4405 complexes in the presence and absence of tapasin. B*4405 is seen to have an improved 37°C stability at 30 minutes compared to B*4402. In the presence of tapasin an improved 37°C and 50°C thermostability is seen for B*4405.

thermostability plots show that in the absence of tapasin there is a reduction in the number of MHC class I complexes over 2 hours. However, within the 4°C stable cohort of molecules, both 37°C and 50°C stable complexes can be identified. This contrasts with the B*4402 pool of complexes. However the time dependent, tapasin independent optimisation is not as great as that seen with B*2705. Upon the introduction of tapasin there is a dramatic change in the stability of the B*4405 complexes. For most complexes the 37°C stability is achieved by 30 minutes whilst there is ongoing optimisation of the 50°C stable cohort. Again when a comparison is made with the B*4402 complexes it can be seen that these are superior to the B*4405 complexes at all time points at both 37°C and 50°C stabilities. Therefore a single point mutation at position 116 is able to alter the loading characteristics of the B*4402 molecule so that it behaves as a relatively tapasin independent allele. This similarity to B*2705 extends to having a dependency upon tapasin for optimal peptide loading, the end product of which is always inferior to that of the tapasin dependent allele B*4402. However the degree to which B*4405 undergoes tapasin independent optimisation is far less marked than that seen for B*2705. Similarly the loss of 4°C material is more noticeable in the absence of tapasin for B*4405 than for B*2705. It is possible that the mechanisms that permit tapasin independent presentation differ between B*2705 and B*4405. Both B*4405 and B*2705 show an early maximal attainment of 37°C stability but never attain total 37°C or 50°C stability. This contrasts sharply with behaviour B*4402. Therefore a trade off is seen between attaining a degree of tapasin independent peptide selection and

achieving a final optimal peptide repertoire. An extension of such studies to other alleles may help delineate the structural/functional features of the class I complexes that influence this behaviour.

Chapter 5 - Discussion

5.1 Tapasin polymorphisms and their genetic and functional relationship

The variable allelic dependency upon tapasin for cell surface expression prompted the initial examination for tapasin polymorphisms. All previous MHC class I allele transfections had been undertaken into a single cell line (721.220) which only expressed endogenous C*0102 (Greenwood et al., 1994). In previous studies of rat MHC class I antigen presentation, a class I modifier (cim) locus had been identified following the study of recombinant strains (Livingstone et al., 1989; Livingstone et al., 1991). This locus had an antagonistic effect on the expression of the rat class I allele RT1-A^a. This locus was shown to encode for a TAP 2 allele (TAP2B) that was unable to supply favourable peptides for RT1-A^a assembly (Powis et al., 1992). The TAP2A allele which had previously been in strong linkage disequlibria with the RT1-A^a allele had been able to supply peptides with a favourable carboxyl-terminal arginine residue, whilst the TAP2B allele was unable to undertake such peptide transport (Momburg et al., 1994; Powis et al., 1996). We wondered whether a similar mechanism might operate for tapasin and were initially encouraged by the description of a second class 1 modifier locus (cim 2) telomeric of TAP (Simmons et al., 1997). (Subsequently this report was retracted by the authors (Taurog et al., 1999)).

Furthermore the existence of certain extended haplotypes (A1- B8-DR3) that exhibited an association with viral diseases (Oksenhendler et al., 1992), such as HIV, promoted us to examine whether alleles of tapasin were maintained in these extended haplotypes. Our initial analysis of the tapasin gene showed a low level of exonic polymorphic variation expected for a gene of approximately 1500bp. (Zhao et al., 2003). In an analogous manner to HLA-DM we found that tapasin showed limited variability in its primary sequence (Sanderson et al., 1994). In regard to HLA-DM, this is believed to be secondary to the role of DM as a chaperone that may operate upon a number of other MHC Class II alleles. Additionally, HLA-DM does not bind peptides and therefore does not require binding pocket polymorphisms to accommodate a specific repertoire of peptides. We would hypothesize that for similar reasons, tapasin exists as a non-polymorphic molecule to maintain its function (or functions) which are applicable to all alleles whose intrinsic polymorphic nature dictates whether they require tapasin assistance or not.

Four notable observations were made following the analysis of tapasin. The first was the corroboration of the coding dimorphism (that had been previously noted (Copeman et al., 1998; Furukawa et al., 1998)) and its frequency, the second was the absence of other exon polymorphisms and the third a description of particular intronic polymorphisms that formed haplotype associations with the coding dimorphism. The fourth observation concerned the absence of a linkage across the MHC class I region. With regard to the coding dimorphism, it is

notable that this mediates a change from an amino acid with a basic side chain (arginine) to that with a polar uncharged hydroxyl side chain (threonine). This charge change may represent an important modifier of function. No functional analysis of the different tapasin alleles upon individual MHC class I allele expression has been undertaken within this thesis. However work undertaken by Momburg and colleagues showed that the tapasin*02 allele was able to restore B*4402 expression to an equivalent extent to that seen in my work utilising the tapasin*01 allele (Tan et al., 2002). The importance (if any) of this dimorphism is not known at present. The genotype frequency of the tapasin alleles within our study population was consistent with the Hardy-Weinburg proportions, with tapasin *01 at 0.53 and tapasin *02 at 0.47. The tapasin*01/*02 heterozygote frequency was 0.49, *01 homozygosity 0.22 and *02 homozygosity 0.28. A study of a Canadian population using PCR restriction fragment length polymorphism of exon 4 with a Bfal digest showed comparable results (Copeman et al., 1998). In contrast, a Japanese population study (Furukawa et al., 1998) showed an *01 and *02 gene frequency of 0.37 and 0.63 respectively (Table 5.1.1). It is not clear if this trend towards a higher frequency of the *02 allele represents a selective functional advantage of this within the common MHC class I alleles of the Japanese population (i.e. A24,B52,B61) or whether it is a reflection of the genetic and racial mix of the island population. All studies so far have been performed on genomic DNA and it would be interesting to investigate whether both tapasin alleles are co-dominantly expressed when different polymorphic variants are present and whether any differences are seen in the ratio of

Tapasin	Williams et al (N=81)	Copeman et al (N=96)	Furukawa et al (N=82)
*01	0.47	0.48	0.37
*02	0.53	0.52	0.63
*01/*01	0.22	0.21	0.13
*02/*02	0.28	0.25	0.39
*01/*02	0.49	0.54	0.48

Table 5.1.1 – Observed frequency of the Tapasin polymorphic variants

The Tapasin *01 allele is arginine (AGA) and the Tapasin *02 is threonine (ACA) alleles.

homozygotes to heterozyogtes in the case of MHC class I associated diseases such as ankylosing spondylitis (AS). If the peptide repertoire of alleles such as B*2705 is important in predisposing some B27 positive individuals towards the development of ankylosing spondylitis (AS), then tapasin may be a candidate gene to influence such a polygenic disorder at a mechanisitic level.

Three novel intronic mutations were identified in intron 4. These were 16147 G→ T, 16232 G→ A and 16317 T→ A. These did not obviously alter potential acceptor/donor splice site regions. The proximity of these polymorphisms to the coding dimorphism permitted an analysis of the exon 4-intron 5 haplotype within each allele. Each coding dimorphism may have been associated with one of eight possible intronic sequences. However, only 3 main haplotypes were identified. For the theronine allele (*02) only a C-GGT haplotype was identified. For the arginine (*01), 3 major haplotypes were identified: G-TAA, G-TGA and G-GGT. Interestingly the GGT intronic sequence was only seen once. This was confirmed on the examination of a further 241 control DNA samples (data not shown). In considering the evolution of the alleles this latter observation might suggest that a G-GGT was originally present and underwent an arginine to threonine (aGa → aCa) point mutation, with subsequent expansion within the population. Why the tapasin *02 haplotype has remained stable whilst the other shows a diversity across the nearby intronic region is not clear. The genetic distance between the polymorphisms is small, varying from 201 bp to the first intronic polymorphism to 351 for the third. Perhaps this tight cis linkage is

evidence of a recent exon 4 mutation that has increased in prevalence and not had time to evolve intronic mutations. This would be supported by the simian data where one finds only the arginine codon (AGA) within exon 4. The absence of a threonine at this position is one of the 15 changes identified between the primate and human tapasin. Furthermore the intron 4 sequence shows only GGT, a configuration that is only rarely seen in linkage with the human arginine tapasin form (*01) but is seen exclusively with the *02 form. If we consider the arginine encoding allele as the ancestral allele for early primates a hypothetical ancestral tree can be outlined (Figure 5.1.1).

The failure to detect a linkage association between the tapasin alleles and the MHC class I region probably reflects the genetic distance between the two loci (2Mb). Although the MHC region shows a lower recombination frequency than expected for a 4Mb region (2% cf 4%) (Thomsen et al., 1994) certain hotspots for recombination exist (Thomsen et al., 1994). Certain areas of the MHC, such those between HLA-B to HLA-C and DQA1 to DRB1, are almost devoid of cross over events whilst others show no marked disequilibria i.e. TAP1 and TAP2. The MHC class II region between DRB1 and DQB1 almost behaves as a single evolutionary unit with extreme linkage disequilibria. However these alleles show little linkage disequilibrium with TAP1 or DPB1, which are located only a short genetic distance centromeric of them. This has been identified as being secondary to a recombination hotspot that exists between TAP1 and TAP2 (Cullen et al., 1995; Klitz et al., 1995). This recombination hotspot may interfere

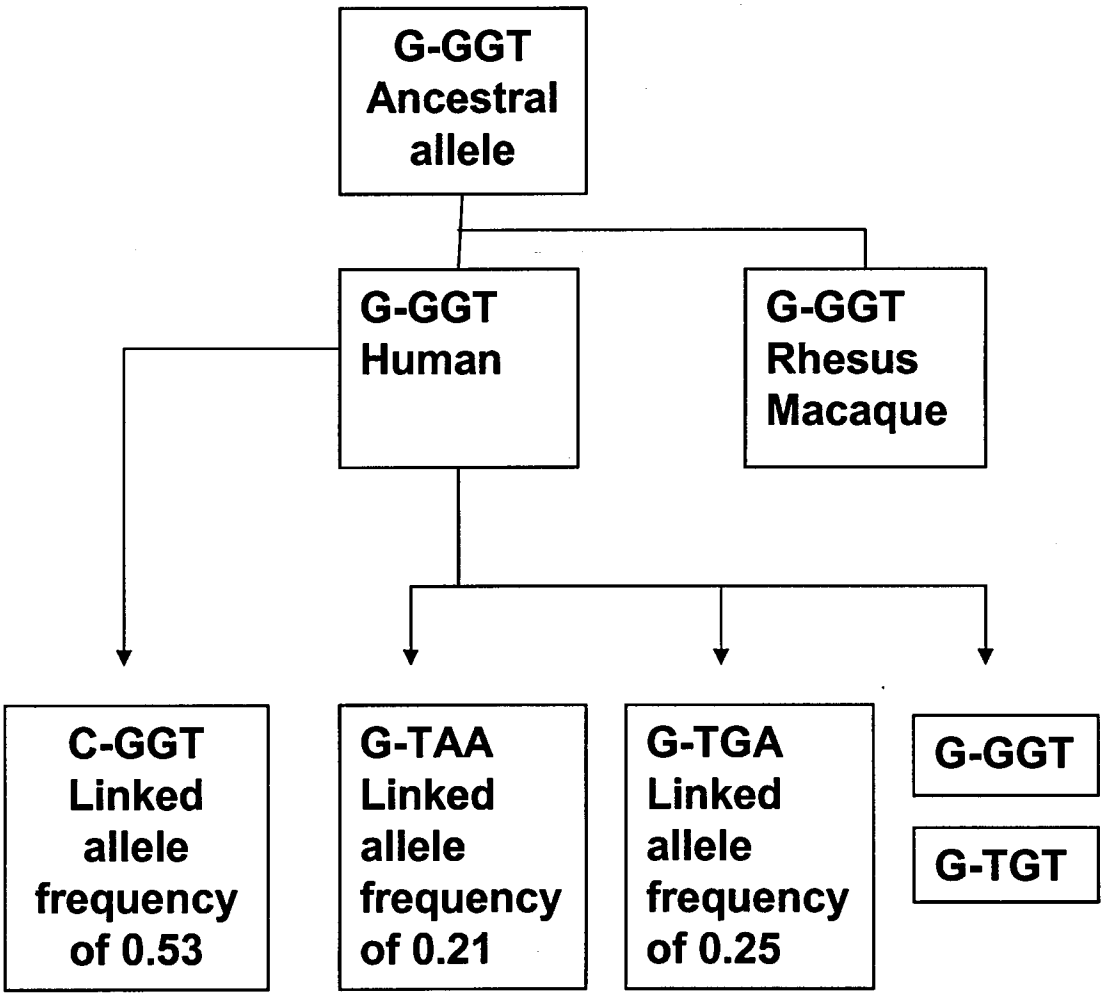


Figure 5.1.1 – Proposed ancestral tree for primate tapasin gene

Consideration of the exon 4 polymorphism and intron 4 dimorphisms across human and simian tapasin sequences permits the construction of an ancestral for primate tapasin

with any cis linkage between MHC class I alleles and tapasin alleles. Within the chicken B locus this situation is very different with the tapasin gene seen within the class II region and located a small distance away from the class I genes. In retrospect the chicken MHC would have been a better genomic target for identifying functionally relevant tapasin MHC cis linkages.

The MHC class II linkage that I observed between DQ6 and DP*0101 with Tapasin*02 suggests that linkage desequiliria may exist centromeric to TAP1. Indeed this region has been termed the extended MHC class II region and shown to have a distinct isochore boundary (Stephens et al., 1999) and exhibit linkage between DPB1 and the KE4 region which is within 80Kb of the tapasin gene (Rajsbaum et al., 2002). The tapasin single nucleotide polymorphisms (SNP's) may permit further linkage studies to be carried out across this extended MHC class II region. Previous studies have suggested an association between a TAP 1 polymorphism (TAP 1.2) and HIV progression and suggested that this association was secondary to a linked gene (Kaslow et al., 1996). It would be now be interesting to analyse whether any of the tapasin alleles are in linkage disequilibrium with TAP 1.2.

The absence of further polymorphisms in the tapasin gene suggested that it may have a common function that could be applied to all alleles. To identify critical regions of tapasin that may be important for this function I choose to sequence primate tapasin from rhesus macaque monkeys. Sequence analysis of 4

macaque genomic DNA samples identified 15 coding differences from human tapasin. This compared with more than 80 differences between human and mouse tapasin (Figure 5.1.2). A comparison of the residues that are different between primate and human tapasin reveals several that are non conservative and 5 which involve a charge change (Table 5.1.2).

Many approaches have been taken towards identifying important functional regions within the tapasin protein.

i) Lehner et al originally deleted the C terminus of tapasin to form soluble protein that was able to restore MHC class I expression but failed to co-localise the MHC class I complex to TAP (Lehner et al., 1998). This was subsequently corroborated by Momburg and colleagues who also showed that the incorporation of the other chaperones was impeded in the absence of the transmembrane region of tapasin (Tan et al., 2002).

ii) Bangia et al showed that the N terminus of the protein was important in associating with the MHC class I complex (Bangia et al., 1999).

iii) Dick et al have recently showed that the cysteine at position 95 is important in the formation of a mixed disulphide interaction with ERp57 and that the abrogation of this interaction prevented incorporation of ERp57 in to PLC (Dick et al., 2002).

iv) Solheim and colleagues have undertaken site directed mutagenesis on murine tapasin and shown that a D337A, S341R or L342T change abrogates a tapasin MHC I class I interaction whilst a H334F and

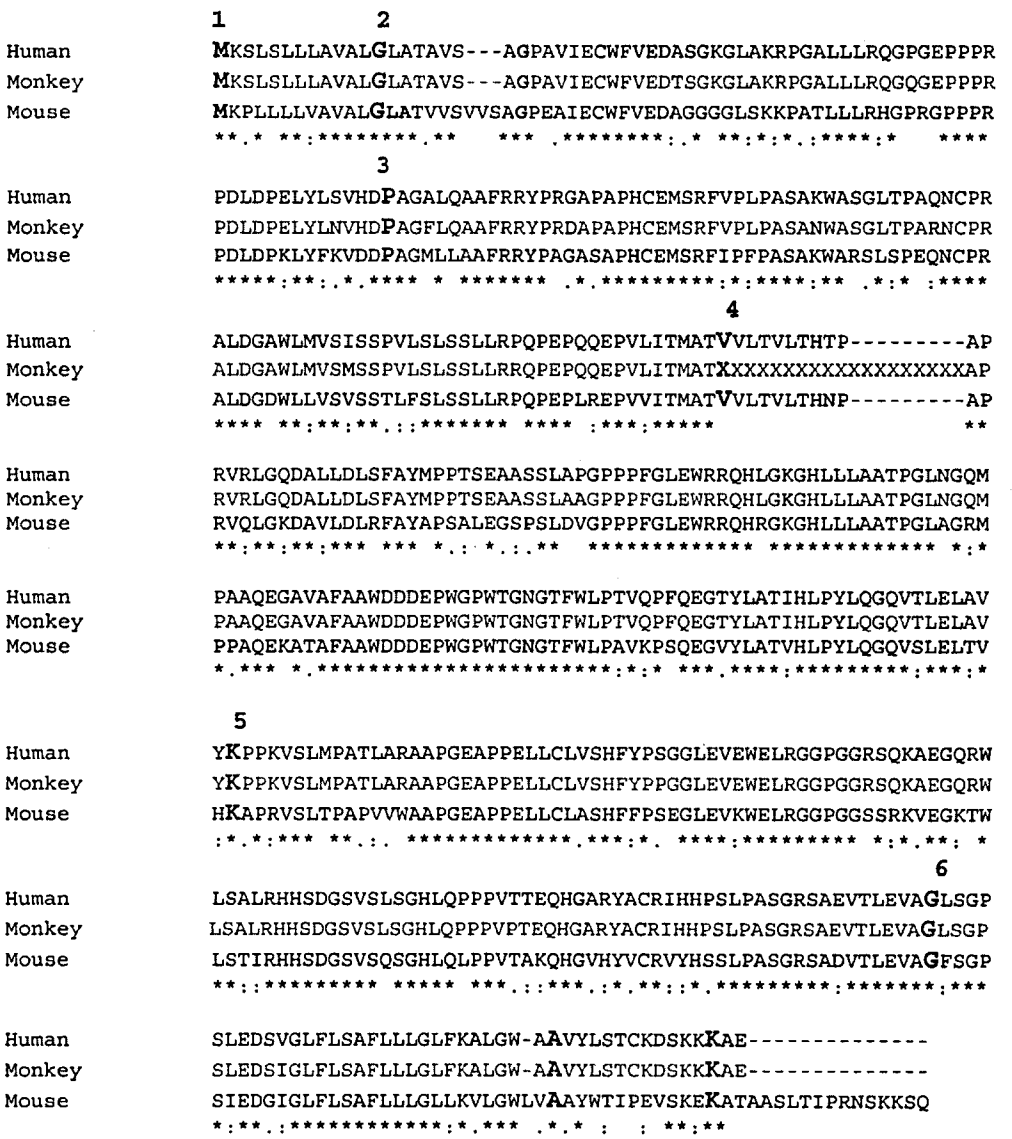


Figure 5.1.2 – Tapasin protein comparison between man, monkey and mouse

The monkey tapasin differs from human tapasin at 15 sites, compared to over 80 for murine tapasin. The * symbol represents amino acid identity and the : symbol disparity between man and mouse/monkey.

Exon	Human (side group)	Monkey (side group)
2	(1)Alanine (Aliphatic and NH)	Threonine (Hydroxl and PU))
	(2)Proline (Imino and NH)	Glutamine (Amide and PU)
	(3)Serine (Hydroxyl and PU)	Asparagine (Amide and PU)
3	(4)Alanine (Aliphatic and NH)	Phenylalanine (Aromatic and NH)
	(5)Glycine (Aliphatic and PU)	Aspartic Acid (Acidic)
	(6)Lysine (Basic)	Asparagine (Amide and PU)
	(7)Glutamine (Amide and PU)	Arginine (Basic)
	(8)Isoleucine (Aliphatic and NH)	Methionine (Aliphatic and NH)
	(9)Proline (Imino and NH)	Arginine (Basic)
4	(10)Proline (Imino and NH)	Alanine (Aliphatic and NH)
	(11)Arginine/Threonine (Basic/Hydroxyl and PU)	Arginine (Basic)
5	(12)Arginine (Basic)	Tyrptophan (Aromatic and NH)
	(13)Serine (Hydroxyl and PU)	Proline (Imino and NH)
	(14)Threonine (Hydroxl and PU)	Proline (Imino and NH)
6	(15)Valine (Aliphatic and NH)	Isoleucine (Aliphatic and NH)

Table 5.1.2 – A comparison of the amino acid differences between human and monkey Tapasin

There are 15 differences between human and monkey tapasin. Those residues with a charge change are highlighted in blue. Abbreviations used are NH- nonploar, hydrophobic and PU – polar, uncharged.

H335Y change retains L^d and reduces the cell surface expression of open form L^d complexes (Turnquist et al., 2001).

The changes identified between human and primate tapasin are currently forming the targets of a tapasin mutagenesis approach to delineate further key areas of the protein that are important in mediating B*4402 expression.

5.2 TAPASIN AND PEPTIDE OPTIMISATION

-DEFINITIONS AND ASSUMPTIONS

The term optimisation is used within my thesis to convey the concept of a MHC class I peptide complex that has a peptide cargo that is able to impart an improved stability of the ternary complex compared to any preceding peptide cargo. Within this framework it is possible to compare the same heavy chain with the same β_2M across at a bulk level and relate changes in thermostability to the nature of the average peptide cargo. We can then compare similar cell lines that differ only in the reconstitution of a single factor that is presumed to be important for MHC class I peptide assembly. We must assume that the restored factor only influences the peptide availability/loadability and does not directly modify the HC or β_2M components of the complex (i.e. facilitated glycosylation/isomerisation). In comparing one allele with another across the same cell line I have been cautious to highlight the sources of error (and of interpretation) in this approach. These have been the different inherent stabilities of the HC/ β_2M interactions in

the peptide vacant state and the possible maximal stabilities for optimally loaded complexes. For both B*2705 and B*4402 early peptide binding experiments illustrated that both complexes could reach maximal stability (50°C) when loaded with optimal peptides. Therefore there was no inherent difference in the maximal stability that could be achieved for different complexes in the presence of optimal peptide. Similarly for B*4405 optimal peptide loading permitted a thermostability of 50°C.

The concept of peptide optimisation is not a new one (Jensen et al., 1999). Indeed it is a requisite for MHC Class II assembly and is believed to principally involve the mechanism of peptide exchange (Vogt et al., 1999). In regard to MHC class I assembly two principal mechanisms may be postulated. The first is peptide exchange and the second is 'intra groove' peptide trimming. The results of my thesis suggest that tapasin is a key component in facilitating optimisation but the precise mechanism has not been addressed. Indeed my experimental work suggests that tapasin may act to improve peptide optimisation both kinetically and qualitatively. In the absence of tapasin all three alleles had a poor thermostability that improved upon tapasin reconstitution. Importantly the impairment of peptide supply, in the absence of tapasin, led to a further deterioration in the thermostability of the complexes. For B*4402 and B*2705 the thermostabilities converged to within 3°C of each other at T50 (temperature point that mediates 50% loss of w6/32 complexes). This suggests that the thermostability differences seen in the absence of tapasin but presence of

peptides represent differential peptide loading of the alleles and not a lowered intrinsic thermo-instability in the absence of peptide. Furthermore the results imply that in the absence of tapasin, this degree of thermo-instability is not reflective of an empty molecule, as it may be further impaired by preventing peptide supply. Thus, in the case of B*4402 its thermostability profile in the absence of tapasin can be seen to reflect either a currently peptide occupied state (suboptimal) or a previously peptide occupied state. This latter idea is at present only a possibility that is consistent with the data and has not been further evaluated. It is possible that in the absence of tapasin, B*4402 is able to load an initial peptide and that this complex is transitory but imparts a change in the thermostability of the complex. The lowered thermostability of B*4402 in the ICP47 transfected cells would then be more likely to represent a truly empty *and* never previously occupied complex. In considering the first option of suboptimal peptide occupation, two further considerations must be taken into account. The first is that such a peptide occupied complex is excluded from ER export. Therefore we have indirect evidence that a MHC class I complex that is occupied by peptide may be subject to quality control at a pre cis-golgi level (all endo H sensitive). The second point is that if B*4402 has the inherent ability to load with peptides why doesn't it behave like B*4405 and B*2705 which also show a similarly low initial thermostability? I suggest that this relates to the abilities of the other alleles to optimise their peptide repertoire independently of tapasin. This property is intrinsic to the alleles and represents (for me) the defining feature of tapasin dependent and independent allelism. However for both B*4405 and

B*2705 their optimisation is kinetically and qualitatively enhanced in the presence of tapasin. Therefore I do not believe that they are entirely tapasin independent alleles. Instead I would consider them to be independent of tapasin for some degree of optimisation but dependent upon tapasin for maximal optimisation. On the other hand B*4402 is entirely dependent upon tapasin for any degree of optimisation. These initial observations may be furthered by considering the following experimental questions. Would B*4402 with a T134K mutation optimise in the presence of tapasin (i.e. unable to interact with PLC)? Would a single chain dimer (B*4402 HC/ β_2M) complex optimise in the absence of tapasin (i.e. increased peptide receptive complex concentration)? Would a single chain trimer (B*4402 HC/ β_2M/optimal. peptide) be independent of tapasin. I am currently exploring such approaches to see if peptide acquisition by B*4402 is truly tapasin dependent or whether it is optimisation of the peptide repertoire that tapasin is required

For B*2705 there was an improvement in thermostability over time in the absence of tapasin. This was seen to a lesser degree with B*4405. This tapasin independent optimisation appears to be a critical factor as it continues to improve during the period of the pulse chase examinations and the cell surface stability of B*2705 was comparable to that of the B*2705 complexes in tapasin competent cells (by radioiodination). This tapasin independent optimisation may also operate through peptide exchange or trimming. A third possibility is that the changing thermostability reflects a biophysical change in the molecule. This idea

is borrowed from the MHC class II field where single peptide occupied MHC class II complexes can change their stabilities over time (Sadegh-Nasseri et al., 1994). The molecular mechanisms underlying this are not clear but may relate to an intramolecular rearrangement of the complex or reconfiguration of the bound peptide into a registry that is more thermostable (Belmares et al., 2003). These processes are highly speculative but are not discounted by the presented data. Indeed, whilst it has been possible to argue that peptide exchange and trimming are the dominant processes driving optimisation within the ER, B2705 continues to improve its thermostability profile after it has left the ER. This is certainly apparent between 60 minutes and 120 minutes in the absence of tapasin. Whilst peptide exchange and/or trimming has been described post ER (Gil-Torregrosa et al., 1998), it is intriguing that this appears to further improve upon the optimisation undertaken in the ER where most of the quality assurance molecules and processes are focused. Trans golgi trimming of peptides by furin has been shown by De Val and colleagues to provide peptides for MHC class I complexes (Gil-Torregrosa et al., 2000). Similarly MHC class I peptide exchange has been described to occur within the endosome for some MHC class I complexes (Gromme et al., 1999; Stryhn et al., 1996). The relative contribution of peptide optimisation occurring outside the ER is an important area that has as yet received little attention. It will be interesting to dissect the contributions of these possibilities with experiments on alleles that are prevented from accessing endosomal compartments (tail mutants/endocytosis inhibition), trapped in a post

golgi/pre cell surface environment (CD99 null cells), or following protease inhibition (i.e. furin inhibition).

THE' ON AND OFF TAP' FOR PEPTIDE LOADING

When tapasin function is restored there is a marked improvement in the thermostability of all 3 alleles. This is most marked for B*4402 with 50°C stability seen for 100% of the loaded complexes. There are 3 important observations to consider when comparing the thermostability profiles in the presence and absence of tapasin.

i) The degree of thermostability achieved by the individual alleles.

ii) The kinetics of thermostability attainment by individual alleles.

iii) The variation in thermostability seen across the different alleles.

For B*4402, there is maximal 37°C stability at 30 minutes and maximal 50°C stability at 120 minutes. This would imply that all complexes have bound a peptide of a relatively high affinity during this time period and is in stark contrast to that seen in the absence of tapasin at any time point. This improved peptide cargo at 30 minutes is also present at 15 minutes when the majority of complexes are endo H sensitive and thus proximal to the medial golgi. The ability of tapasin to influence this enhanced thermostability may be through a number of different direct and indirect effects. As the keystone of the PLC, tapasin may simply recruit the peptide receptive (or sub optimally loaded complex) to the PLC where ERp57/CRT and enhanced peptide availability mediate an improved peptide selection (Momburg and Tan, 2002). The

individual contribution of tapasin to such a cooperative assembly is difficult to dissect. It is possible to return peptide supply to normal without co-localising the MHC I class I complex to the PLC (N terminal deletion of tapasin) (Bangia et al., 1999). Similarly it is possible to remove tapasin from the TAP based PLC (C terminal deletion of tapasin) (Lehner et al., 1998; Tan et al., 2002). When the former of these was undertaken there was no improvement in optimisation. This suggests that the ability to influence TAP level and peptide transport is not directly relevant to the optimisation observations made in this human EBV transformed B cell model (in contrast to mouse tapasin deficient cells (Grandea et al., 2000) (Garbi et al., 2003)). However when soluble tapasin was transfected, a significant change in the thermostability profile of B*4402 was seen. Although the stability at the end of the chase period was comparable to full length tapasin at 37°C, it was reduced at 50°C. Additionally there was no loss of 4°C material in the presence of soluble despite not all complexes being 37°C stable. Thus soluble tapasin was able to permit optimisation for B*4402 but the extent of this process was less than that of full length tapasin. This may relate to a need to be near the TAP complex from optimal peptide selection, the reduced affinity of class I for soluble tapasin, a differential recruitment of chaperones by soluble tapasin or even an impaired trimming when MHC class I is not part of the PLC complex. However it is important to note that the inability of B*4402 to become properly loaded with optimal peptide in the absence of tapasin was restored with soluble tapasin without any effect upon peptide supply. This is an important observation as it suggests B*4402 peptides are present in the absence

of a TAP/tapasin complex but cannot be utilised for B*4402 (in contrast to B*2705) in the absence of tapasin. Therefore tapasin can be considered to have at least 2 functions. One is to co-localise MHC class I to the PLC and the other is to promote peptide stabilisation and optimisation of the MHC class I complex. This optimisation is maximal when both functions are coordinated. Consideration of the work of Momburg and colleagues is relevant at this juncture (Tan et al., 2002). They looked at the expression of B*4402 in the presence of a soluble tapasin construct and focused upon the chaperone associations. They were able to identify tapasin:MHC class I complexes that were not bound to calreticulin or ERp57. They were also able to show that B*4402 was associated with an altered peptide repertoire, with a number of different assays, but total cell surface expression of B*4402 was normal. Therefore the optimising function of tapasin is unlikely to be the ability to facilitate the cooperative assembly of the other chaperones on TAP. This action may be a composite of optimal chaperone recruitment plus an additional function which has not yet been clearly identified. This additional function may relate directly to peptide exchange, peptide trimming or an action upon the MHC class I complexes to make it more receptive to the above processes.

POST ER OPTIMISATION

The kinetics of optimisation are different between soluble and full length tapasin, with soluble tapasin facilitating maximal 37°C stability at 120 mins compared to 30 mins for full length tapasin. What is interesting is the observation that 50°C

stability, whilst quantitatively different for both, plateaus between 30 and 60 minutes and then further improves for both alleles between 60 and 120 minutes. This is the time period when the majority of complexes have left the ER. Therefore I would suggest that there are 2 phases of optimisation and improved thermostability. The first is within the ER and may be entirely tapasin dependent for certain alleles. For alleles such as B*4405 and B*2705 tapasin independent optimisation may occur. In the presence of tapasin such alleles have the choice of utilising or bypassing the tapasin dependent optimisation process. In comparing the profiles of B*4402 with those of B*2705 and B*4405 in the presence of tapasin, it may be suggested that the poorer profiles of the latter two alleles (lower 50°C plateau) is a result of both tapasin independent and dependent ER processes being used. The former complexes may be of a poorer affinity leading to a reduction in the net thermostability of those alleles. The second phase of optimisation would appear to be post cis-golgi and contribute to the conversion of 37°C stable to 50°C stable complexes. The nature of this process would appear to be operating for all alleles in the presence of tapasin (and to a similar relative degree) but only for B*2705 in the absence of tapasin. Whilst tapasin-facilitated optimisation (Opt 1) prevents complex loss within the ER and promotes the rapid attainment by empty complexes of 37°C stability, later optimisation (Opt 2) permits a further improvement in the peptide loaded material. This division of optimisation is the starting point for the analysis of what would appear to be different processes of MHC class I complex peptide optimisation.

MECHANISMS OF OPTIMISATION

For optimisation 1 tapasin may act in a number of ways.

1) Tapasin as a peptide exchange catalyst

Tapasin may act like HLA-DM upon low affinity occupied class I complexes and destabalise suboptimally loaded MHC class I complexes. This 'destructive' approach may be secondary to an interaction with the MHC class I complex that is permissible when suboptimal peptide is present but not inhibited when optimal peptide is bound. Such a mechanism would appear to require a structural difference between the sub-optimally loaded and optimally loaded complex to enable tapasin to distinguish between the variants. Such differences have not been identified through crystalisation studies on extended peptide MHC class I complexes (Khan et al., 2000) or short peptide MHC class I complexes (T Elliott ,unpublished observations). If there is no structural difference between peptide occupied complexes of differing stabilities perhaps tapasin acts upon conserved peptide/MHC class I interactions and that this association promotes destabalisation of the peptide MHC class I complex. If for example tapasin interacted with the MHC class I complex and this interaction destabalised the peptide-MHC class I interaction, peptide release may be precipitated. The likelihood of peptide release would then relate to the inherent stability of the peptide MHC class I interaction and the kinetics of the interaction. Such a peptide release mechanism may be dependent upon a tapasin induced conformational change in the MHC class I structures which would not be

identified in crystalisation approaches to MHC class I structures. Such a conformational change may be secondary to the destruction of certain key peptide main chain/MHC class I groove hydrogen bonds and the establishment of a open peptide receptive conformer, as has been suggested for HLA-DM (Mosyak et al., 1998). Although this proposed action would form the basis of a molecular mechanism to explain an iterative improvement in selected peptides, it does not explain why B*4402 is unable to sample its high affinity peptides in the absence of tapasin.

2) Tapasin as a peptide trimming facilitator

At present, there is no experimental evidence on the role of tapasin and ER aminopeptidase activity. The recent identification of ERAP as an important trimmases of MHC class I peptides will now allow such an assessment. It is conceivable that tapasin promotes N terminal trimming indirectly through recruitment of ERAP to the PLC or directly through a cooperative action with ERAP. Again the inability of B*4402 to load with peptides in the absence of tapasin within this conceptual framework would suggest that the majority of B*4402 peptides are extended or do not have canonical anchors and thus are poorly loaded. The change in the thermostability between ICP47+/- cell lines may support such a proposal as the difference between the cell lines would reflect the loss of extended peptides. It may then be hypothesised that 220/ICP47 complexes are empty and transport-incompetent 220 complexes occupied with suboptimal peptides are transport-incompetent, and 220/tapasin complexes being optimal and transport-competent. This supposition will require

further examination but I think it unlikely for the following reasons. ERAP has been shown to be most essential to the mouse allele L^d which has a preference for proline at P2. As proline at this position is poorly transported by TAP, extended peptides are preferentially transported for this allele. B*4402 has no such restriction. Furthermore when ERAP is knocked down in L^d cell lines the degree of downregulation is greater than that seen in the absence of tapasin (Serwold et al., 2002). A dependence upon tapasin for trimming would predict a comparable or worse phenotype in the absence of tapasin. However the contribution tapasin makes towards a trimming process may account for the improved optimisation profiles seen for B*2705 and B*4405. The N terminal extension of a single amino acid can reduce the thermostability of a complex considerably and this may have an important impact upon the final stability of the MHC Class I peptide complex (Cerundolo et al., 1991; Dedier et al., 2000).

3) Tapasin as a 'doorman' for the MHC class I complex

Tapasin may act to prepare (hold open the door) the MHC class I complex for peptide acquisition. It may also return a complex that has undergone a conformational change after binding a suboptimal peptide to a peptide receptive form (open the door again). Furthermore it may select which peptides permit groove closure by altering the triggering threshold of the open complex such that energetically disfavourable suboptimal peptide induced groove closures are avoided (offering the revolving door for unwanted visitors). Although hypothetical, such a mechanism may account for allelic differences in the dependence of tapasin for peptide loading. Alleles such as B*4402 may undergo

groove closure with suboptimal peptides (low threshold) and require assistance to reopen and try again. Alleles such as B*2705 may have a higher threshold and only trigger closure with peptides that permit more favourable hydrogen bond interactions or have the intrinsic ability to return to a peptide receptive conformation in the absence of chaperone assistance. We have previously likened this to a Venus fly trap where closure of the plants leaves only occurs when sensitive hairs at the base of the trap and at some distance from one another are touched two or three times, offering a means of kinetic proofreading (Williams et al., 2002). This composite signal for closure, ensures that larger insects are caught rather than the smaller insects that would only trigger one or two areas of the plant base. This 'quality control' is important as this process is energetically costly, as the plant is unable to return to a functional open form for a further 3 days following closure. HLA-DM has also been postulated to have a stabilising function on the MHC class II complex, facilitating the maintenance of a peptide receptive complex. It is not clear at a molecular level how this operates and whether a continued interaction or transient interaction with the MHC class II complex is required (Pashine et al., 2003). It is intriguing to speculate that convergent evolution may occur and that such processes may be conserved across antigen presenting molecules and may even involve comparative systems for glycolipids/CD1 associations

4) Tapasin as a 'conformational catalyst'

One assumption that is made in the interpretation of increasing thermostability being representative of an evolving peptide repertoire is that there is no other

factor that improves the thermal denaturation characteristics of the MHC class I peptide complex. In the presence of tapasin it may be argued that the improved thermostability represents a facilitated foldase activity upon a peptide occupied complex that improves its cooperative stability (Li et al., 1997). I think this is unlikely for a number of reasons. Firstly it has been previously demonstrated that peptide alone alters the cooperative stability of the ternary complex within in vitro systems (Cerundolo et al., 1991; Elliott et al., 1991; Parker et al., 1992). Secondly the B*2705 complexes show an improvement in thermostability in the absence of a tapasin interaction. Thirdly the alleles can be seen to improve their thermostability at a time period when they would not be expected to be in contact with tapasin. However, again with reference to HLA-DM, it has been argued that DM facilitates the evolving stability of MHC class II complexes through its action as a conformational catalyst (Verreck et al., 2001; Zarutskie et al., 2001). Further definitive work in this area requires the preparation of a recombinant tapasin which has currently eluded all investigators.

For optimisation 2, I see three possibilities that may operate beyond the ER. The first is peptide exchange, probably occurring within an endosomal or cell surface location (Schirmbeck et al., 1995; Schirmbeck and Reimann, 1996). What is difficult to explain within this context is how B*4402 is able to undertake such an exchange independent of tapasin outside of the ER when it was unable to achieve this in the absence of tapasin within the ER. Recent studies have suggested that a tapasin facilitated loading is necessary for subsequent vacuolar

presentation to occur (Chefalo et al., 2003). Therefore optimisation 2 may be tapasin independent but tapasin is needed to get B*4402 molecules out of the ER. The environment of the endosomes may then permit tapasin independent peptide exchange. What factors promote or inhibit peptide exchange in the endosomes and ER respectively in the absence of tapasin requires further investigation. The second possibility is one of ongoing peptide trimming that occurs en-route to the cell surface or when the complexes are undertaking their cell surface-endocytic cycle. (Gromme and Neefjes, 2002; Schirmbeck and Reimann, 2002). Experimental inhibition of these processes may be possible to further refine this concept. Finally the same peptide complex may undertake a conformational change which imparts an enhanced thermostability profile upon the complex. The final stability of the complex will be the product of the initial peptide and the enhanced stability conferred upon the ternary complex by such a conformation change. Whether such a 'twist' exists is currently hypothetical but may now be addressed through the examination of trimeric single chain constructs that only have a single optimal peptide (Yu et al., 2002). These complexes would be expected to have an identical thermostability profile throughout their ontogeny as they have a covalently attached optimal peptide. Examination of such complexes within cells may provide valuable information of the nature of optimisation 2.

5.3 DM – COMPARISONS AND CONTRASTS

The work presented in my thesis draws many parallels with previous work undertaken on the MHC Class II chaperone HLA-DM (DM). DM is encoded within the MHC class II locus and has been shown to be non-polymorphic. Its crystal structure has been solved and shown to have a similar fold and domain structure to that of classical MHC class II molecules (Fremont et al., 1998; Mosyak et al., 1998). Importantly DM contains two novel disulphide bonds in the region homologous to the peptide binding site of DR and possesses none of the pockets for binding the side chains of peptides. In DM, the α helices corresponding to the 'sides' of the peptide binding groove of classical class II complexes contact each other, precluding peptide binding . The crystal structure of tapasin has not been solved and modelling of its structure has shown closest phylogeny with that of a MHC class I structure. This modelling has been extended by Jan Klein to suggest that tapasin may have a structure of a modified MHC class I molecule, once again drawing a comparison with DM (Mayer and Klein, 2001). These comparisons at best tentative and we await the crystal structure of tapasin with interest.

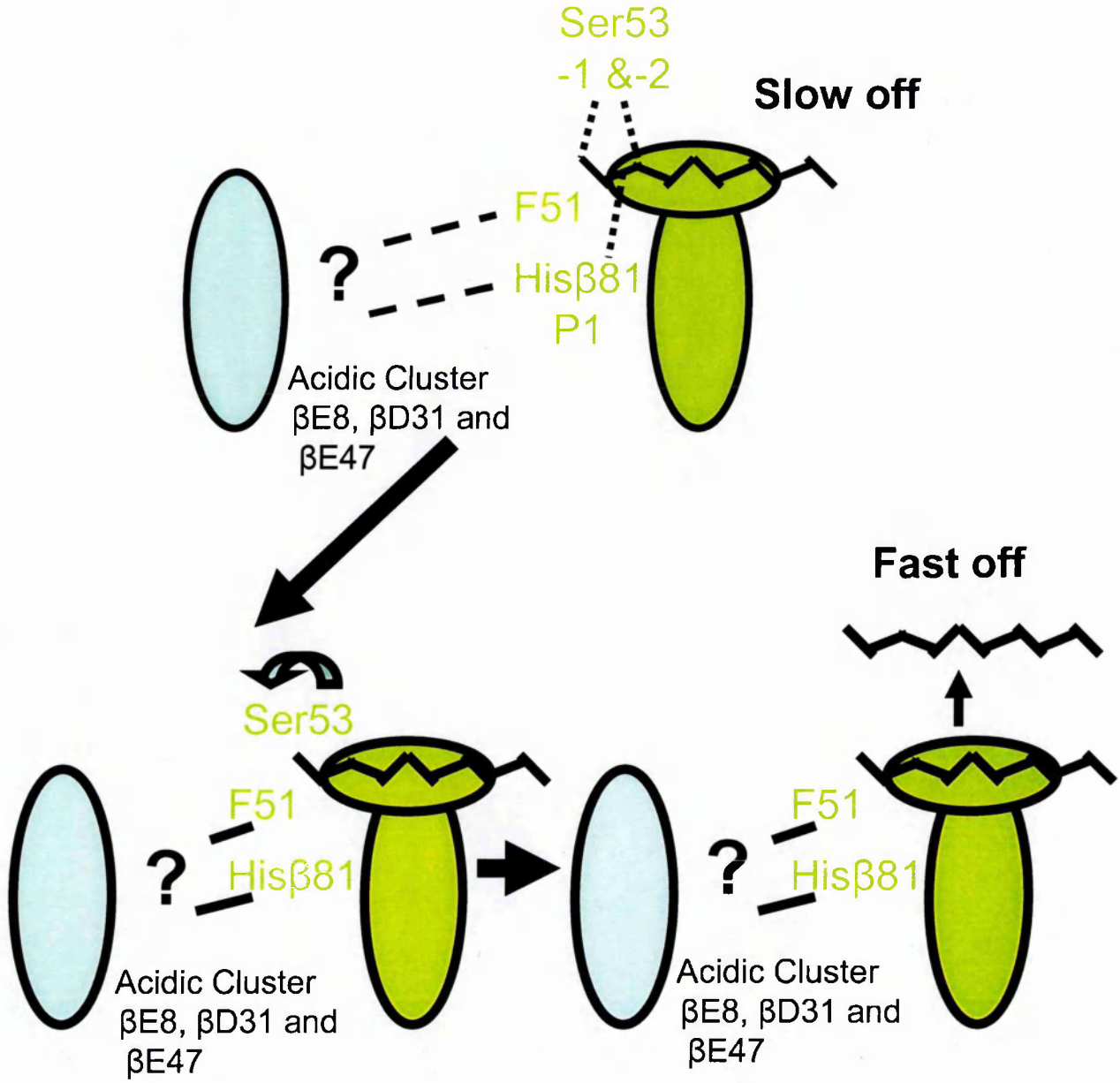
Tapasin appears to be multifunctional for MHC class I assembly.

1. It is clearly needed to stabilise TAP (Garbi et al., 2003) and this is dependent upon the transmembrane region of the molecule (Lehner et al., 1998).
2. It is absolutely required to permit assembly of the MHC/ β_2M heterodimer/calreticulin/ERp57 complex at the TAP interface. In the absence of tapasin, MHC class I complex binding to calreticulin is reduced. Certain alleles (such as HLA-A2) cannot be co-immunoprecipitated with calreticulin (Lewis et al., 1998), whilst other alleles such as HLA-B27 and D^d can be identified using sensitive immunoprecipitation and western blot protocols (Harris et al., 2001; Paquet and Williams, 2002).

In addition to these two functional properties there is much discussion with regard to other proposed roles. I would like to suggest from my work that tapasin has an important role in optimising the repertoire of peptides bound to MHC class I complexes. This property is in addition to that of peptide supply and MHC class I co-localisation to the PLC as evidenced by the soluble tapasin experiments. This peptide optimisation is reminiscent of the role DM plays in MHC class II assembly. Before considering the way in which tapasin mediates peptide optimization let us consider the experimental data accrued on DM peptide optimisation.

The role of DM in peptide editing was first described in 1995 by Denzin and Cresswell (Denzin and Cresswell, 1995). Using imuno-purified DM, DR and a specific reagent for suboptimally loaded DR (CerCLIP) they identified 2 key features of DM function. The first was that it liberated CLIP from the suboptimally loaded DR and that it permitted peptide loading at the appropriate pH of the MIIC. Furthermore, when compared to a detergent that liberated CLIP and permitted peptide exchange it was shown to improve upon the rate and efficiency of such loading compared to this detergent. This was furthered in 1996 by the experiments of Weber and Jensen (Weber et al., 1996). They showed that DM was able to dissociate other peptides from DR and that the enhancement of DM mediated dissociation was directly proportional to the intrinsic dissociation rate of the peptide. Work by Kropshofer and Hammerling identified an important influence of P1 anchor mutants of the haemagglutinin (HA) peptide for a DR/DM interaction (Kropshofer et al., 1996). They suggested that suboptimal residues at the P1 anchor position may be a key molecular signature for DM interaction. Allelic differences in DM dependence were noted with the mouse allele I-E^k showing little dependence upon DM for peptide loading (Wolf et al., 1998). A model for DM activity has been proposed whereby DM may selectively disrupt hydrogen bonds between the MHC class II molecules and the main chain atoms in the bound peptide. Peptides with optimal anchor residues remain relatively stable whereas those with suboptimal anchors (such as CLIP) dissociate. Publication of the structure of DM in 1998 provided further support for a model in which DM acts to break (or compete for) hydrogen bonds between the MHC

class II molecule and bound peptide near the peptide N terminus at P1. Hydrogen bonds between CLIP and DR3 involve important contributions from Histidine (H/His) β81 and Serine (S/Ser) α53 of DR3. These resides are outside of the binding groove and were candidates for an interaction with the lateral surface of DM. Initially it was postulated that the DMα chain tryptophan (W/Trp) α62 of DM might interact with the Phenylalanine (F/Phe) α51 of DR3 and move the neighbouring Ser α53, thereby breaking 2 conserved hydrogen bonds at P-2 and P-1 (Mosyak et al., 1998). A further interaction with His β81 of DR3 may distort the third hydrogen bond of P1 (Figure 5.3.1). However more recent mutagenesis studies have shown that D61R and W62A mutations in DMα do not impede the function of DM whereas mutations in a conserved cluster of acidic residues in DMβ severely impaired both CLIP release and peptide binding (Pashine et al., 2003). Interestingly, MHC class II structural comparisons have shown that I-E^K has a shallow P1 pocket and this may relate to its difference upon DM dependence (Fremont et al., 1996). These ideas have been extended by the groups of Sant, McConnell and Sadegh-Nasseri. Sant and colleagues analysed the murine class II molecule I-A^d which bound peptides in the absence of strong pocket interactions. A single mutation at His β81 showed that disruption of a single hydrogen bond severely impaired the stability of the peptide MHC complex (Arneson et al., 2001). The allele I-A^k was subsequently examined as, in contrast to I-A^d , it engaged peptides through strong pocket interactions. An identical His β81 mutation led to rapid degradation of the complex compared to wild type (McFarland et al., 1999). This could be



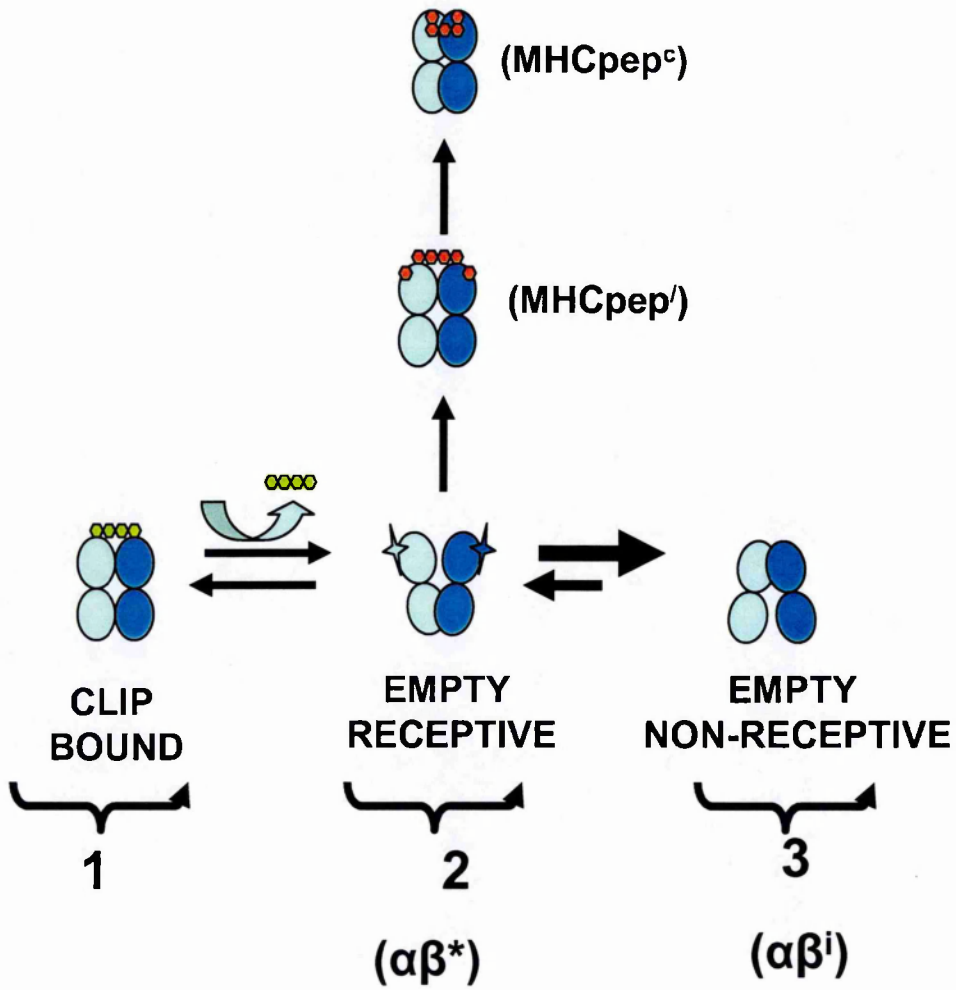
5.3.1 – Speculative molecular mechanism of action of HLA-DM on HLA-DR

DM may act upon the residues of the DR groove to break important hydrogen bond networks between the peptide and α/β chains of the class II molecule (-1,-2,P1). This may then favour liberation of the peptide from the binding groove

prevented by stabilisation with high affinity ligand but an association with DM could not be identified. Work by Sadegh-Nasseri and McDonnell suggested that empty class II complexes could exist in at least 2 conformations (Sadegh-Nasseri and McConnell, 1989). The first conformation was peptide receptive and was that seen immediately after peptide/CLIP dissociation. In the absence of peptide binding it would rapidly convert to a second conformer that was resistant to peptide binding. This second isomer slowly converted to the peptide receptive form over time (Sadegh-Nasseri and Germain, 1991; Sadegh-Nasseri et al., 1994). Work by McConnell suggested that an importation function of DM was to maintain the class II complex in the peptide receptive conformation (Rabinowitz et al., 1998). Sadegh-Nasseri has further suggested that DM may favour the conversion of the peptide non-receptive conformer to the peptide receptive conformer. Finally, Stern and colleagues have proposed that DM has an important role post peptide acquisition (Zarutskie et al., 2001). They have followed up on the work of Sadegh-Nasseri et al who had shown that MHC class II single peptide complexes were more stable the longer they were in contact with each other and that the initial MHC peptide complex ($MHCpep^{/}$) may progress over time to a more compact and stable complex ($MHCpep^{c}$). Stern and colleagues have proposed that DM acts as a conformational catalyst to promote the conversion of $MHCpep^{/}$ to $MHCpep^{c}$. Furthermore they showed through UV circular dichroism, gel filtration and hydrodynamic radii estimation that conversion from an empty to a conformed state may occur with a variety of partial groove occupancies. These conformational states show loss of antibody recognition for

an open form of class II, increased thermostability and different dichroism spectra. These same changes may be induced through occupation of only the P1 pocket or P5-P9 regions. However, only when all interactions are optimum is there maximal dichroism, thermal stability and SDS stability. Such overlapping interactions that promote the conversion to a compact form may be analogous to those occurring in the transition of an optimally peptide loaded $MHCpep^{I}$ to $MHCpep^{C}$ complex (Figure 5.3.2).

How similar is tapasin to DM in its functions within MHC class I assembly? Notable differences include the belief that MHC class I complexes do not have a CLIP equivalent and that peptide vacant complexes are all peptide receptive. However suboptimal peptide loading has been proposed as an important initial event in MHC class I assembly, perhaps acting in an analogous manner to CLIP (Lewis and Elliott, 1998). My work would support the existence of a suboptimally loaded MHC class I complex that may precede the loading of optimal peptides. The thermostability differences seen between B*4402 and B*2705 +/- ICP47 would be consistent with suboptimally loaded complexes that may subsequently change their peptide cargo. Prior to the start of my experimental work it was suggested that tapasin associates with peptide receptive complexes that are devoid of peptide and mediates a loading advantage for peptide that had been previously available but not utilised. This facilitated loading process appeared to occur equally well when MHC class I and tapasin were not bound to TAP and therefore not dependent upon a high local concentration of peptide. However as



5.3.2 - Proposed actions of HLA-DM

1 – Release of CLIP : 2 – Maintenance of class II complex in a peptide-receptive confirmation: 3 – Conversion of empty non-receptive class II complex to the peptide-receptive conformer: 4 – Loading of peptide and conformational change to a compact stable class II complex

shown by my work (and that of others (Tan et al., 2002)) the bridging function to TAP does convey a different repertoire of peptides upon that allele. This advantage is not simply due to enhanced availability of peptides but due to the ability of tapasin to optimally direct peptide loading when associated with the PLC. The inability to load previously available peptides is most clearly seen for the mouse H2-M3 allele and HLA-G (Chun et al., 2001; Park and Ahn, 2003). In these experiments high affinity peptides are not bound by the alleles in the absence of tapasin. Such complexes remain in a compartment proximal to the medial golgi mannosidase enzymes and do not exit until they are furnished with such peptides. Despite their inability to load high affinity peptides that may liberate them from the ER, their ER half lifes are much longer than similar β_2M conformed complexes in peptide transport incompetent cells. This raises the intriguing possibility that these complexes are or have been loaded with suboptimal ligand and that such an interaction permits an MHC class I extended lifespan, allowing for the eventual capture of an optimal peptide. My work suggests that B*4402 is not devoid of peptide in 721.220 as its thermostability is further reduced in the presence of an ICP47 blockade of peptide transport. This is akin to the nonclassical allele associations above, requiring tapasin to permit loading of available high affinity peptides. Experiments using an N terminal deletion of tapasin that is able to restore maximal TAP transport but is still unable to support B*4402 expression, argue against peptide availability as an alternative reason for poor loading. How could tapasin be mediating this loading? With analogy to DM, tapasin may bind to MHC class I complexes that have a

suboptimal ligand. Tapasin may then permit liberation of the suboptimal ligand. Two new considerations arise from this idea. The first is that previously occupied MHC class I molecules may exist in 2 forms, one of which is peptide receptive and the other of which is not. Tapasin may influence this ratio and stabilise peptide receptive forms in a way proposed for the action of DM in promoting an $\alpha\beta^*$ form that can be readily loaded (Rabinowitz et al., 1998). The second consideration is that tapasin may 'fix' on a structural motif such as exposed hydrophobic residues or a signature motif such as a glycosylation signal for a molten globule state. With regard to these considerations:

1) There is notable allelic variance in the association of MHC class I molecules with tapasin. This has been observed with natural variants and selected mutants (see Section 5.4) and has focused principally on position 116. My work with B*4405 is consistent with this pattern where this residue determines the tapasin dependence of loading. Thus the configuration of the F pocket may be a signature for tapasin binding. It is possible that certain alleles have an F pocket configuration which promotes a peptide receptive conformation and does not require tapasin to stabilise such a conformer. Alternatively, tapasin dependent alleles may have a different F pocket configuration which favours a peptide induced conformational change even following transient peptide occupancy (Fig. 5.4.2). Tapasin dependency may then relate to the property of tapasin to target such alleles that are of a peptide non receptive conformation and return them to a peptide receptive state. This

would be analogous to DM promoting an $\alpha\beta^{i} \rightarrow \alpha\beta^{*}$ conformer for peptide loading in class Ii assembly. Alternatively such tapasin dependent alleles may have a binding groove anatomy that is relatively difficult to induce into conformational change with suboptimal peptide. Tapasin dependent alleles such B*4402 may undergo a conformational change that is dependent upon F pocket interaction/peptide interaction even when other pocket/peptide interactions are unfavourable. Experiments with the mouse allele D^b and the peptide FAPGYNYPAL lend some support to this idea (Elliott and colleagues, unpublished observations). Truncated C and N terminus peptides (FAPGY and NYPAL) have differential effects upon the conformational changes of D^b. Within in vitro assembly assays both 'half peptides' are required to drive a conformational change in D^b that allows for immunoprecipitation by a conformationally sensitive antibody. However the C pocket directed suboptimal NYPAL peptide is able induce a full conformational change in D^b when co-crystalised with D^b. The structure of the suboptimally loaded complex is almost identical to that of the optimally loaded D^b complex apart from the occupation of the N terminus pockets with water and glycerol. This may be likened to the properties of a Venus fly trap as mentioned earlier where the apex of the trap should not be triggered by small prey, requiring multiple signals from the 'groove' to induce a productive closure. Position 114 contributes to the groove residues of the F and E pockets and it will be interesting to see whether this alters the configuration of the empty class I structure between

the 2 molecules. The approach of Sadegh-Nasseri and colleagues with the MHC class II DR3 allele is interesting with regard to these pocket changes. They engineered a DR3 complex with a shallow P1 pocket which is predicted to be a poorer target for DM despite the inability to load stabilising peptides (Natarajan et al., 1999). Indeed this $DR1_{\beta G86Y}$ mutant has a number of interesting properties that are relevant to the class I alleles B*4402/*2705 and B*4405. Firstly the empty complex has an improved midpoint of thermal denaturation compared to wild type (72°C vs. 65°C) and also showed improved SDS stability, an assay format previously considered representative of stably occupied complexes (Sato et al., 2000). Further work has suggested that SDS stability reflects the degree of exposure to SDS of hydrophobic residues in the P1 pocket. It is speculated that DM acts through conformational recognition of a 'floppy' class II configuration and that this is representative of class II that is empty or loosely bound to peptide in the P1 pocket (Chou and Sadegh-Nasseri, 2000). In the case of the $DR1_{\beta G86Y}$ mutant, the P1 pocket is made artificially 'rigid' and therefore, despite a low affinity peptide load, it is not seen by DM. Whether an analogous process is occurring within the F pocket of MHC class I complexes is intriguing. It would be interesting to measure the thermostability of the 114 mutant complexes made in the absence and presence of a supply of peptides. My predication would be that the B*4402D114H would be more thermostable than B*4402 within an

ICP47 transfected cell line, maintaining a peptide receptive form and with a reduced tendency for conformational change with suboptimal peptides.

2) The second consideration is whether tapasin has specific recognition of a signature motif within the MHC class I complex and whether glycosylation at position 86 in the HC is important for this recognition. Interesting studies on murine class I have shown that inhibition of deglucosylation (through castanospermine [CAST] treatment) traps MHC class I complexes onto tapasin (van Leeuwen and Kearse, 1996). Furthermore studies by Sadasivan and colleagues showed that CAST prevented human MHC class I association with tapasin but did not impair heterodimer formation (Sadasivan et al., 1996). It was initially postulated that this may relate to an inability to bind a Glc_1 residue to calreticulin which is required prior to tapasin association. An alternative is that tapasin requires a Glc_1 signature for association. Recent work has shown that tapasin association with TAP and class I is not affected in the absence of calreticulin (Gao et al., 2002). Furthermore in the absence of tapasin a MHC/ β_2M /calreticulin association is very difficult to see (Harris et al., 2001). It is therefore possible that the Glc_1 motif in the α1 domain of class I is a marker of an incompletely folded complex and that tapasin is able to register this and initiate binding with subsequent recruitment of calreticulin to this N86 monoglucosylated conformation. If a peptide binds to the complex a conformational change may occur which lowers the affinity of calreticulin for the heterodimer. Glucosidase II may then trim the

monoglucosylated glycan upon the PLC and prevent its re-association with calreticulin, permitting the release from tapasin and the PLC. If this is not possible, calreticulin may rebind to the PLC and stabilise the peptide loaded complex on the PLC. A projection of this concept is that PLC associated class I in calreticulin knock out cell lines should be more transiently associated with tapasin. The faster trafficking rate of K^b in the absence of calreticulin would support this prediction. Tapasin dependent alleles that are engineered to lack an N86 glycan (B*4402-/-/-) or that are trapped in a Glc_3 state (B*4402 +CAST) may fail to utilise tapasin facilitated loading due to inability to associate with the peptide loading complex. Studies by Salter and colleagues showed that a B*0702 S88A mutant which is non glycosylated fails to interact with calnexin and is expressed on the cell surface at 50 % of wild type (Zhang and Salter, 1998). Targeted experiments that selectively inhibit glucosidase II (butyl-deoxynojirimycin) or impair UGGT (RNAi) may offer new approaches to these questions.

Finally, does the suggestion that DM may act as a conformational catalyst have relevance to the mechanism of action of tapasin? A seminal study by Sadegh-Nasseri examined the stability of recombinant DR1 complexed with a radiolabelled peptide. The study showed that there existed both a fast on/off and slow on/off interaction with the same peptide. Furthermore the conversion to a slow on/off rate complex was time dependent, changing from a DR1:peptide $t_{1/2}$

of 10 mins following 10 mins incubation to 140hr after 96 hours incubation. It was postulated that peptide bound MHC class II complex could exist in 2 conformers, one compact and stable and the other flexible and unstable. Subsequent work by Stern and colleagues suggested that DM acted to convert the peptide bound intermediate (MHC-pep$^{/}$) to the more compact stable form (MHCpep). It was argued that DM acts to stabilise the transition intermediate, thereby favouring interconversion. A further complication has been recently proposed by McConnell and colleagues who have suggested that DM can differentially catalyse a CLIP peptide DR*0404 complex with regard to 2 isomers having different peptide registries within the peptide binding groove. A second interpretation is that each registry imparts a conformational change which does not change the monophasic dissociation of the complex at pH7 but does provide a signature for DM mediated biphasic dissociation at pH5. Does this have any relevance to the actions of tapasin? The observation that B*4402 can undergo a time dependent improvement in its thermostability requires a consideration of this phenomena. Although peptide exchange and peptide trimming are the most likely explanations for this improvement a tapasin facilitated conformational mechanism should be considered. The interconversion of MHC-pep$^{/}$ to MHCpep complex following tapasin assisted loading may account for the improved thermostability profile post ER egress. Schmidt and McConnell have reported such a peptide class II complex whose conformer shows differential sensitivity to DM mediated dissociation at pH5.3 but identical dissociations at pH7 (Schmitt et al., 1999). More recently Hammerling and colleagues have identified a DR/CLIP

complex that shows differential reactivity with an antibody in the presence or absence of DM. It was suggested that this conformation was independent of peptide but dependent upon a prior interaction with DM (Verreck et al., 2001). Previous studies by McCluskey and colleagues on B*2705 and Momburg and colleagues on B*4402 have demonstrated differential antibody reactivities between tapasin competent and incompetent cells (Purcell et al., 2000; Tan et al., 2002). It is probable that these represent different peptide repertoires but it will be interesting to look at such cell lines with peptide specific antisera to see if there are possible conformational differences in bound complexes in the absence or presence of tapasin.

Therefore the role of tapasin in MHC class I assembly is analogous to HLA DM in many ways. It is multifunctional, allele dependent, non-polymorphic and improves the rate and extent of MHC stability. Whether it can release suboptimal ligands, stabilise and favour the formation of peptide receptive isomers and act as a conformational catalyst for peptide occupied complexes remains to be determined

5.4 TAPASIN AND MHC CLASS I ALLELE DEPENDENCE/INDEPENDENCEALLELIC

The original description of the tapasin deficient cell line 721.220 commented upon a post translational, allele specific process that was absent in this cell line

(Greenwood et al., 1994). Further work subsequently identified tapasin as the missing factor and a hierarchy of allele dependence/independence based upon cell surface expression levels was described (Li et al., 1997; Ortmann et al., 1997; Sadasivan et al., 1996). Grandea et al and Braud et al extended these allele dependences to include the non-classical complexes of HLA-G and E respectively (Braud et al., 1998b; Grandea et al., 1995). Peh and colleagues first described the alleles B*4402 and B*2705 as representative of the extremes of tapasin dependence and independence respectively (Peh et al., 1998). These serial observations have all approached the question of what determines tapasin dependence at both a functional and structural level. My initial approach within this work was to explore whether B*4402 and B*2705 were significantly different as peptide receptive complexes with regard to their stability and peptide receptivity. One explanation for tapasin dependence might have been a requirement of a chaperone interaction for the stability of a peptide receptive complex prior to peptide acquisition. B*4402 may have required such an interaction and B*2705 not. However examination of the complexes in the presence of ICP47 when they are as "empty" as possible showed very little difference in their thermostability profiles, with neither showing expression at the cell surface. Therefore an intrinsic difference in the affinity of the HC:β_2M interaction that permits a tapasin independent loading phenotype does not seem likely. When one looks at the primary sequence of the tapasin dependent and independent alleles many differences are present. In comparing B*4402 with B*2705 there are 20 differences, occurring within the α_1, α_2 and α_3 regions

```
          1                                                          60
B*2705   ,GSHSMRYFHTSVSRPGRGEPRFITVGYVDDTLFVRFDSDAASPREEPRAPWIEQEGPEYW
B*4402   ,GSHSMRYFYTAMSRPGRGEPRFITVGYVDDTLFVRFDSDATSPRKEPRAPWIEQEGPEYW
          ******** *  ***************************** *** ***************
          61                                                         120
B*2705   ,DRETQICKAKAQTDREDLRTLLRYYNQSEAGSHTLQNMYGCDVGPDGRLLRGYHQDAYDG
B*4402   ,DRETQISKTNTQTYRENLRTALRYYNQSEAGSHIIQRMYGCDVGPDGRLLRGYDQDAYDG
          ****** *   ** ** *** ************  * ***************** ******
          121                                                        180
B*2705   ,KDYIALNEDLSSWTAADTAAQITQRKWEAARVAEQLRAYLEGECVEWLRRYLENGKETLQ
B*4402   ,KDYIALNEDLSSWTAADTAAQITQRKWEAARVAEQDRAYLEGLCVESLRRYLENGKETLQ
          ********************************** ****** *** *************
          181                                                        240
B*2705   ,RADPPKTHVTHHPISDHEATLRCWALGFYPAEITLTWQRDGEDQTQDTELVETRPAGDRT
B*4402   ,RADPPKTHVTHHPISDHEVTLRCWALGFYPAEITLTWQRDGEDQTQDTELVETRPAGDRT
          ****************** ****************************************
          241                                                        300
B*2705   ,FQKWAAVVVPSGEEQRYTCHVQHEGLPKPLTLRWEPSSQSTVPIVGIVAGLAVLAVVVIG
B*4402   ,FQKWAAVVVPSGEEQRYTCHVQHEGLPKPLTLRWEPSSQSTVPIVGIVAGLAVLAVVVIG
          ************************************************************
          301                                  338
B*2705   ,AVVAAVMCRRKSSGGKGGSYSQAACSDSAQGSDVSLTA
B*4402   ,AVVAAVMCRRKSSGGKGGSYSQAACSDSAQGSDVSLTA
          ************************************
```

Position	9	11	12	41	45	67	69	70	71	74
B*2705	H	S	V	A	E	C	A	K	A	D
B*4402	Y	A	M	T	K	S	T	N	T	Y

Position	77	81	94	95	97	114	156	163	167	199
B*2705	D	L	T	L	N	H	L	E	W	A
B*4402	N	A	I	I	R	D	D	L	S	V

Figure 5.4.1 – Comparison of the primary sequences of B*4402and B*2705

The 20 differences between B*4402 and B*2705 are seen in red. Identical aspartic acid residues (D) at position 116 are highlighted in blue.

(Figure 5.4.1). The 20 differences are mostly within the α_1, α_2 regions with a single change in the α_3 region. The 116 position is identical between the 2 alleles and highlighted in blue. When one compares B*4402 and B*4405 there is only a single difference at position 116, a negatively charged aspartic acid in B*4402 (D116) and an aromatic tyrosine (Y) in B*4405. Position 116 is an important residue as it, in addition to other residues (Table 5.4.1), forms part of the F pocket which accommodates the C terminal peptide residue.

How might a single residue change the dependence upon tapasin for the acquisition of peptide? The motif for B*4402 is E at P2 and Y/F at P9. I would suggest that the change at 116 crucially affects the function of the F pocket for these alleles. Ascribing a function to a pocket of the peptide binding groove other than its role in dictating specificity is tentative at present. I would suggest that the substitution of a negatively charged residue for an aromatic residue at this position, whilst not directly impacting upon the specificity of the F pocket could change the propensity of the MHC class I molecule to undergo a peptide induced confirmation change. The D>Y change enables B*4405 to bind to relatively high affinity peptides in the absence of tapasin help. This may relate to a structural change in the hydrogen bonding network that is set up with the peptide receptive molecule. It is possible that this change also permits B*4405 to remain peptide receptive following low affinity peptide interactions or it may increase the threshold level of any peptide induced conformational change. It is also possible that the altered F pocket permits an improved transition between

Pocket	Residues	Peptide position accommodated
A	5,7,59,63,66,99,159,163,167,171	1
B	7,9,24,35,34,45,63,66,67,70,99	2
C	9,70,73,74,97	6
D	99,113,114,155,156,159,160	3
E	97,114,147,152,156,159,160	7
F	77,80,81,84,95,116,123,143,146,147	Carboxy-terminus

Table 5.4.1 – Amino acid composition of the MHC class I peptide binding groove pockets

The 6 peptide binding pockets that are constitute the peptide binding groove of MHC class I HC have contributions from several amino acids. Each pocket accommodates a particular amino acid residue of the bound peptide.

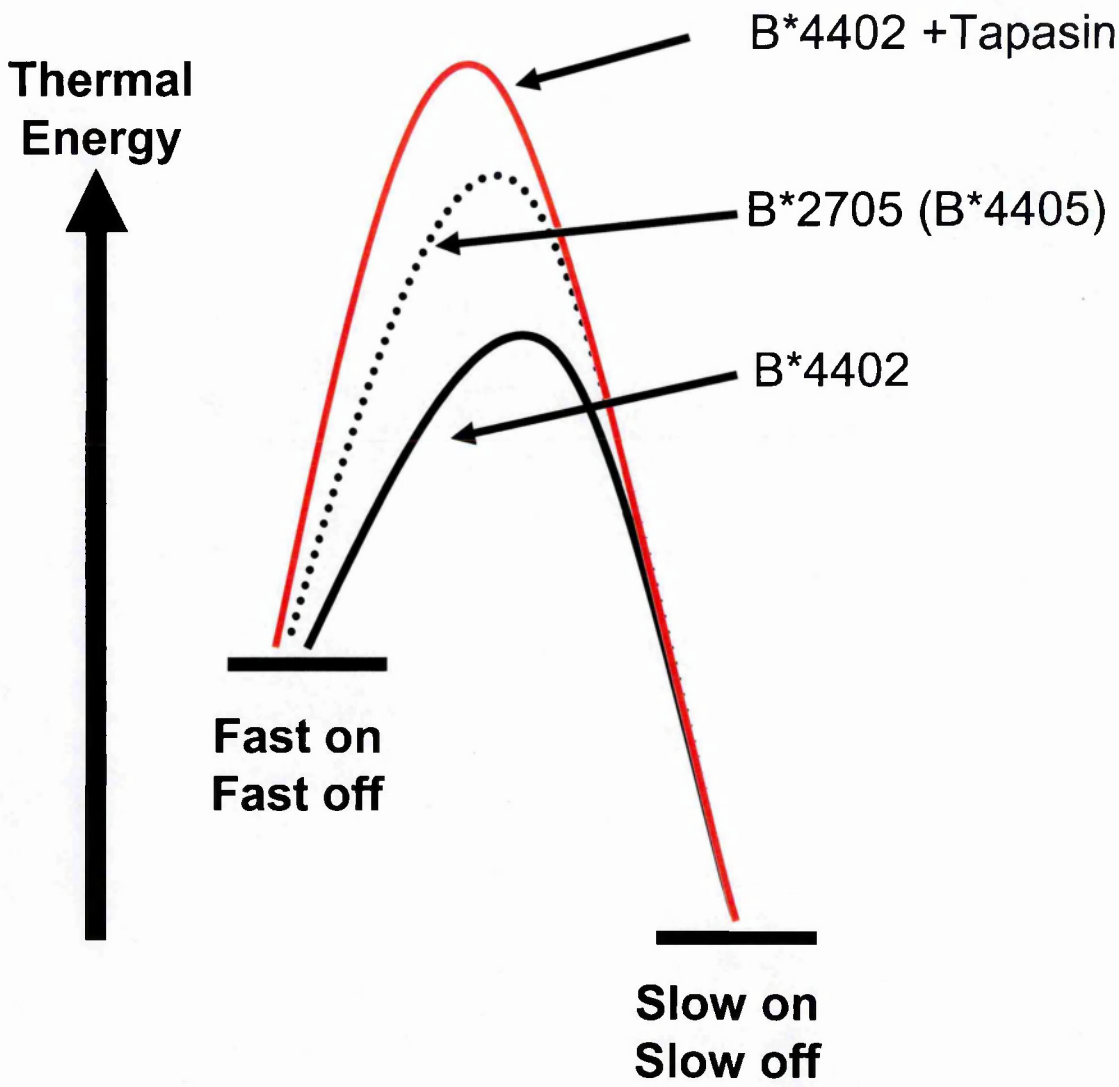


Figure 5.4.2 – Hypothetical model peptide induced conformational changes in different MHC class I complexes

The conversion of a peptide receptive complex to an occupied one will need to overcome an energy barrier for such a conformational change to occur. This change may be induced through a F pocket peptide interaction. This may be more easily triggered with lower affinity ligands for alleles such as B*4402. Tapasin may resist such low affinity peptide mediated conformational changes.

peptide non-receptive and receptive states (Figure 5.4.2). The importance of position 116 had been previously eluded to regarding the ability of alleles to interact with the TAP/tapasin PLC complex. Alleles such as B*3501 with a serine (small polar) at 116 bound poorly to TAP whilst B*3503 which only differed from B*3501 by the substitution of phenylalanine (aromatic) at 116 was able to associate with TAP (Neisig et al., 1996). Turnquist and colleagues have studied the alleles B*1510 (Y116) and B*1518 (S116) which only differ at a single residue. Again the aromatic amino acid at 116 permitted a good interaction with TAP whilst the serine did not (Hildebrand et al., 2002). For A*6807 (H116) a single change to D116 (A*68012) abolishes a Tap association (Turnquist et al., 2002). However, in B*0701 the mutagenesis of Y116 to D116 greatly improves the TAP association (Turnquist et al., 2000). Again the tapasin dependences of these alleles and their mutants would be interesting, with the prediction that those showing a TAP association would be more tapasin dependent. Most recently, work by Ahn and colleagues has implicated a single amino acid within the α_2 domain (114) as a critical determinant of tapasin dependence (Park et al., 2003). They identified a number of alleles that were tapasin independent (B*2705, B*2702, B*0801 and B*5401) and some that were tapasin dependent (B*4402, B*3501, A*3001 and HLA-G) (Table 5.4.2). Those alleles that were tapasin independent all had a histidine (basic) at position 114 whilst those dependent alleles possessed a negatively charged glutamic acid (E) or aspartic acid (D). Remarkably when B*4402 was mutated (D114H) it became tapasin independent whilst B*2705 (H114D) became tapasin dependent. Furthermore

Pocket Contribution	C B	F	F	F	E C	D B A	E D	F	E D	P9 Peptide
Residue	70	77	81	95	97	99	114	116	156	
Dependent										
B*4402	N	N	A	I	R	Y	D	D	D	**Y/F**
B*3501	N	S	L	I	R	Y	D	S	L	**Y/F**
A*3001	Q	D	L	I	I	Y	E	H	L	**Y/L**
G*0101	H	N	L	L	W	I	E	Y	R	**L**
E*0101	T	N	L	L	W	H	E	F	Q	**L**
A*0101	H	N	L	I	I	Y	R	D	R	Y
B*2705 H114D	K	D	L	L	N	Y	D	D	Y	?
Weakly Dependent										
B*0801	N	S	L	L	S	Y	N	Y	D	L
B*5401	Q	S	L	W	T	Y	N	L	L	A
B*4402 D114N	N	N	A	I	R	Y	N	D	Y	?
B*2705 H114N	K	D	L	L	N	Y	N	D	Y	?
Independent										
B*2705	K	D	L	L	N	Y	H	D	L	R/K
B*4405	N	N	A	I	R	Y	D	Y	D	Y/F
B*2702	K	N	A	L	N	Y	H	D	L	**F/Y**
A*0201	H	D	L	V	R	Y	H	Y	L	**L**
A*2401	H	N	A	L	M	F	H	Y	R	**F/W**
B*4402 D114H	N	N	A	I	R	Y	H	D	D	?

Table 5.4.2 - Pocket residues of Tapasin dependent and independent alleles

The important amino acid residues of the F pocket are highlighted in yellow. Other amino acid residues that are represented in at least 2 other pockets are also shown. The preferred Carboxy terminus anchor residues are highlighted in boldface. The alleles highlighted in red (A*0101 & B*4405) have position 114 residues that do not conform to the notion of an acidic residue at this position conferring tapasin dependence and a basic residue, tapasin independence.

the basic 114 B*4402 complex showed a lowered affinity for tapasin whilst the negative 114 B*2705 complex demonstrated a higher affinity for tapasin compared to their natural alleles. This approach has since been extended to the non classical HLA-G allele (Park and Ahn, 2003). Position 114 is interesting as it contributes to the D and E pockets of the peptide binding groove (Table 5.4.1). However B*4405 has an aspartic acid at position 114 and is independent whilst A*0101 has a basic arginine at position 114 and is tapasin dependent (Greenwood et al., 1994). In considering all these experimental findings it is clear that broad generalisations about single residue alterations and predicted functional consequence are likely to be allele specific and relevant within the context of the structure of that particular complex. However the attention upon the F pocket and possibly the E pocket places an emphasis on the interaction of the C terminal and P7 residues of the peptide. Perhaps in the context of B*4402 and B*2705 the 'tapasin-independent inducing' mutations alter peptide selection so that peptide loading may occur with the similar set of peptides, due to the relaxation of certain functional constraints that may have been previously present. Similarly for the 'tapasin-dependent inducing' mutations a structural constraint for a productive F pocket-peptide-interaction may have been invoked, thereby limiting assembly with the previously favourable peptide cargo.

I consider that the allele differences in the requirement of tapasin for peptide loading will relate to a structurally determined property of each allele that facilitates their chaperone independent peptide receptivity. Certain alleles will be

peptide receptive following early assembly and following a transitory peptide induced conformational change. This permits multiple cycles of peptide assembly/disassembly without loss of peptide receptivity until a high affinity peptide is loaded. For dependent alleles this acquisition of peptide either requires tapasin to enable peptide receptivity following early assembly or following the conformational change secondary to a low affinity peptide interaction. Thus tapasin would both increase the quantity of peptide receptive complexes (a quantitative component of tapasin function) and control the quality of loaded peptide for such alleles (a qualitative component of action). The mutations mentioned probably alter the balance of these chaperone requirements for each allele. The characterisation of such molecular alterations will probably require crystalisation approaches to the various allele variants, analysis of the peptide vacant forms and possibly the assessment of other MHC class I mutants that selectively target intra-hydrogen bond networks within the peptide binding grooves.

5.5 Summary - integrated pathways of peptide loading, optimisation, trafficking and cell surface expression.

The molecular mechanisms and exact differentiation pathways of MHC class I assembly still elude us. Two major assumptions may be made concerning the assembly and export of MHC class I complexes. The first is that the process of

ER assembly and export of this heterotrimeric glycoprotein complex share many similarities with other transmembrane multi-subunit glycoproteins. The second assumption is there are specific 'regulations' placed upon some MHC class I complexes that may relate to their subunit assembly, i.e. - peptide abundance/suitability, β_2m affinity, HC anomalies or chaperone interactions. The work undertaken in this thesis and its relationship to the existing literature has been assembled into the following working model.

- THE MHC HEAVY CHAIN → GLYCOPROTEIN FOLDING AND OXIDATION

The unfolded HC is glycosylated at an asparagines (Asn) residue (position 86) within the α1 domain of all human class I proteins. The growing polypeptide chain is post translationally modified on an NXT/s glycosylation sequon as the nascent chain is translocated to the luminal side of the ER. A 14-saccharide 'glycan unit' is transferred from the dolichylpyrophosphate carrier to the Asn residue via an N-glycosidic bond (Figure 5.5.1). This triglycosylated oligosaccharide ($Glc_3Man_9GlcNAc_2$) subunit has an important role in the folding of nascent glycoproteins and this is of equal importance in MHC class I assembly. It has been previously noted with other protein models that the glycosylated regions of a protein are not random but coordinate with tertiary structures that have important conformational properties (Ellgaard and Helenius,

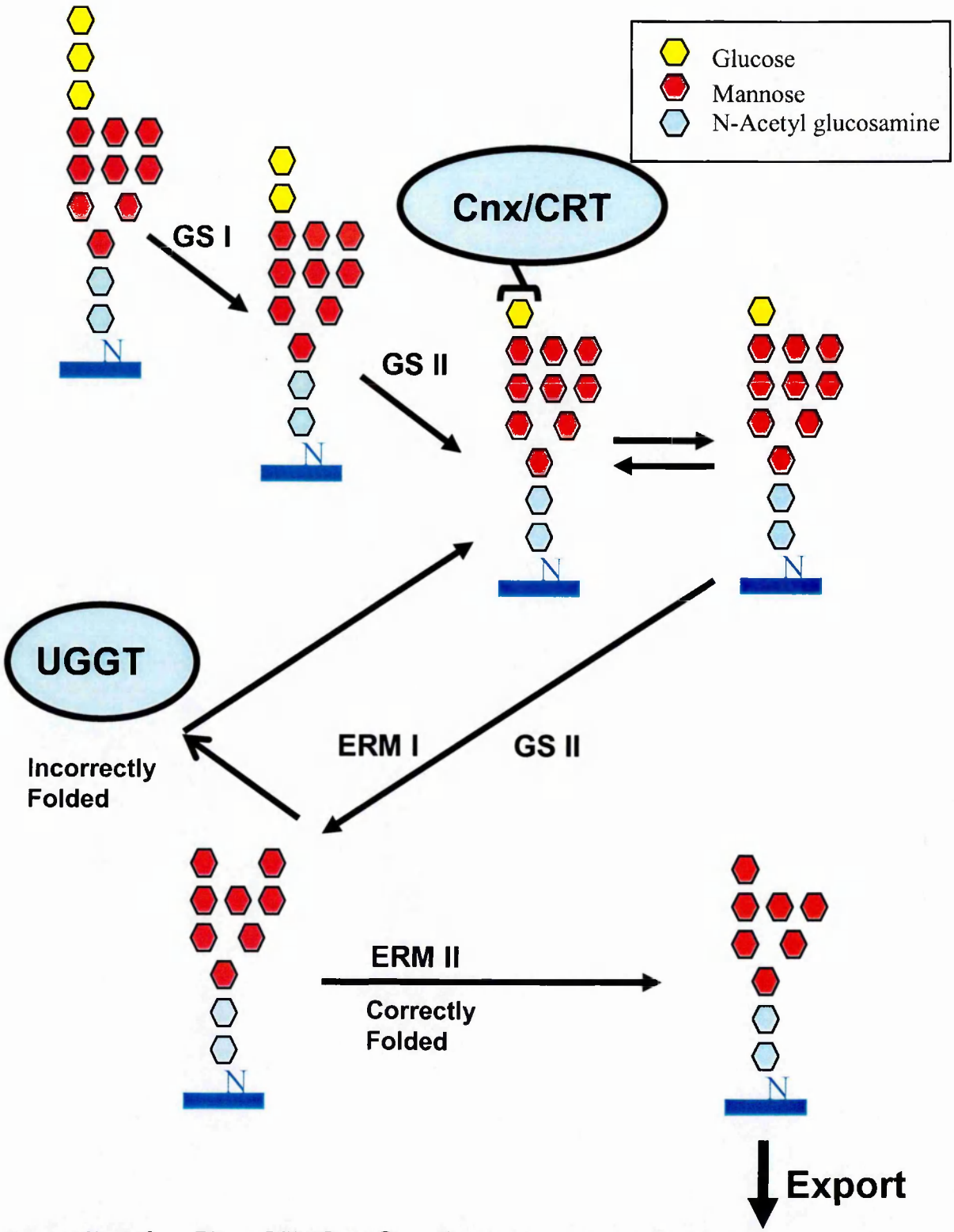


Figure 5.5.1 – The MHC HC – Glycoprotein folding

The first stage in the assembly of a heterotrimeric MHC class I complex is the folding of the glycosylated HC. This is undertaken by the chaperones calnexin and calreticulin. β_2 m binding to oxidised HC permits release from calnexin and suitability for calreticulin binding or GS II cleavage. UGGT reglucosylate the HC for a further cycle of folding.

2003). This is of importance with regard to the α1 region of the class I molecule and I will return to this point later. The $Glc_3Man_9GlcNAc_2$ subunit is immediately acted upon by ER resident glycosidases (GS 1 and II). The membrane bound GS I removes the terminal glucose and the residual 2 glucoses are removed by the soluble GS II. Further glycan processing may proceed within the ER through the action of mannosidases I and II to generate a $Man_7GlcNAc_2$. Besides deglucosylation and demannosylation a further glycan modification may occur within the ER. This is the re-glucosylation of the previously glycosylated mannose by the enzyme UDP-glucose:glycoprotein glucosyltransferase (UGGT). Its activity is dependent upon the integrity of the polymannose core with greater activity for the $Man_9 > Man_8 > Man_7$ structures (Frigerio and Lord, 2000). This appears to be of importance for 3 reasons. Firstly, the UGGT has been shown act as a protein folding sensor acting to re-glucosylate proteins that are incompletely folded (Caramelo et al., 2003). Secondly, monoglucosylated proteins are a substrate for the ER resident chaperones calnexin and calreticulin which have an important role in the assembly of MHC class I complexes. Thirdly, the glycan side chain and in particular its glucosylated and mannosylated structure are important in mediating the removal and degradative pathway of ER glycoproteins (ERAD) (Tsai et al., 2002).

The calnexin-calreticulin cycle is an important 'quality control' step in the formation of many glycoproteins. These homologous proteins have been extensively investigated in the processes of glycoprotein quality control and

specifically MHC class I assembly. The principle is that monoglucosylated $Man_{7-9}GlcNAc_2$ subunits are ligands for both calnexin and calreticulin. Removal of the terminal glucose disrupts this interaction leading to dissociation, whereas re-glucosylation by UGGT would permit a further cycle of interaction. Calnexin was initially described as associating with mouse MHC class I molecules (Degen and Williams, 1991). This association was shown to be increased in cells where MHC class I assembly was impaired through peptide or β_2M deficiency. The role of the glycan in mediating this interaction has been looked at in 3 ways. Firstly, HC point mutants have been made which replace asparagines (N86) residues (Zhang et al., 1995), secondly enzyme inhibitors have been used to prevent glucose trimming to the monoglucosylated form (Neefjes and Ploegh, 1988) and thirdly calnexin deficient cell lines have been generated (Scott and Dawson, 1995). Zhang and Salter investigated the human A*0201 (A2) allele by transplanting an additional glycosylation site at position 176 within the alpha 2 domain (Zhang and Salter, 1998). Two important points were made. Firstly the double glycosylation mutant bound more stably to calnexin compared to wild type A2 and secondly the mutant bound to calnexin in both a β_2M associated and free form. This latter point supported earlier work which had identified a division between mouse and human class I assembly where mouse calnexin was able to remain associated with β_2M bound heavy chain whereas human heavy chains were unable to do so (mouse class I heavy chains are all glycosylated at position 86 and 176) . A model was constructed where human class I associated with calnexin during its initial folding utilising position 86 and upon correct folding and

β_2M association, release from calnexin occurred with subsequent availability of the monoglucosylated N86 to soluble calreticulin but inaccessibility to calnexin (Sugita and Brenner, 1994). In comparison, mouse still posses glycans at positions 176 (+/- 256) and may simultaneously co-associate with both calreticulin and calnexin (Nossner and Parham, 1995). However this association may not be strictly dependent upon glycan interactions as Carreno and colleagues generated an aglycosylated mouse L^d allele and showed comparable association with calnexin compared to wild type L^d . However, the cell surface expression of this allele was reported to be much lower than that of the wild type allele with more peptide receptive complexes identified within the ER (Carreno et al., 1995). In mannosamine treated cells the glycan chain is prematurely terminated as a $Glc_3Man_{5-7}GlcNAc_2$ subunit (Bennett and Kearse, 1999). The mouse allele K^b was shown to associate poorly with calnexin when this termination was induced whilst its association with calreticulin was comparable to wild type K^b. In tunicamycin treated human cells, which completely inhibits N-linked glycosylation, allele differences were found in the assembly of class I alleles. For many B locus alleles there was a failure to associate with β_2M and an enhanced binding to antibodies that detect unfolded class I molecules (Neefjes and Ploegh, 1988). For certain class I alleles (mainly A locus) only small differences were seen in the presence or absence of glycosylation. Finally MHC class I expression has been assessed within a calnexin deficient human cell line. No differences were seen in the trafficking rate, heavy chain turnover or cell surface expression of MHC class I molecules between mutant and wild type

cells. This may be due to the redundancy of function between folding chaperones in the ER. Indeed, some groups have shown that the protein BiP may be important in HC folding or degradation (Nossner and Parham, 1995; Paulsson et al., 2001b). It is not clear whether BiP is involved in targeting unfolded heavy chains for degradation or facilitating folding.

These studies demonstrate that early MHC class I assembly is similar to other glycoproteins, utilising the glycoprotein modifications of the ER to assist in folding, quality control and degradation in the absence of a correct structure. These process may show allele and species selectivity in their generalised actions upon MHC class I assembly. In what way do the glycoproteins assist in these processes at a molecular level?

The calnexin-calreticulin cycle has an important role in the mechanism of UGGT based folding but is also thought to be crucial for the recruitment of thiol oxidoreductases (High et al., 2000). The oxidising environment of the ER and the presence of thiol oxidoreductases permit disulphide bond formation within the ER. MHC class I. complexes have a disulphide bond in the alpha 3 domain (cys203-cys259) and within the alpha 2 domain (cys101-cys164). One of the thiol oxido reductases , ERp57, is particularly interesting in the context of MHC class I assembly. ERp57 is a member of the protein disulphide isomerase family (PDI) with 2 thioredoxin motifs that constitute its thiol/disulphide oxidoreductase sites. It is of interest in the assembly of MHC class I complexes as it is recruited to

glycoproteins to participate in corrective folding by the calnexin-calreticulin cycle. This was shown by Oliver et al where blockade of lectin binding prevented ERp57 mediated disulphide formation (Oliver et al., 1997). Furthermore, release from ERp57 was only permitted upon deglucosylation. For MHC class I assembly 3 groups reported an association between ERp57 and class I heavy chain (Hughes and Cresswell, 1998; Lindquist et al., 1998; Morrice and Powis, 1998). This was identified in rat, mouse and human systems. Important points from these papers were that the ERp57 was seen within the peptide loading complex, tapasin was required for ERp57 incorporation into the complex, a 'sub' loading complex could exist in the absence of TAP and inhibition of glucose trimming prevented co-association of ERp57 with MHC class I HC. It has proved more difficult to identify early complexes of MHC class I HC with calnexin/calreticulin outside of the PLC. Previous studies undertaken by Bulleid et al required crosslinkers to demonstrate the co-association of the glycoprotein with the calnexin/calreticulin/ERp57 complex (Farmery et al., 2000). Lindquist et al probed for a mixed disulphide intermediate through the use of a membrane permeable alkylating agent to trap mixed disulphides (Lindquist et al., 2001). Using an antibody to the free HC (in a detergent that disrupted the PLC) they identified a 105kDa protein which was shown to be ERp57 and MHC HC. Furthermore, they showed that an antibody to free heavy chain could co-immunoprecipitate ERp57 in a calnexin positive cell line but not in calnexin negative cell line. As calnexin is not part of the human PLC this implies co-association in the early assembly of MHC class I HC. Interestingly in the

calnexin deficient cell line ERp57 was not seen as a mixed disulphide with HC but ERp72 was seen instead. Such redundancy of function possibly accounts for the normal class I expression in CEM-NKR cells. More recently, recombinant ERp57 has been shown to form mixed disulphides with HLA-B*2705 (Antoniou et al., 2002). This interaction has preference for partially folded HC and may promote further unfolding with subsequent targeting for degradation. This again underlies the dual function of the early glycoprotein chaperones in the 'quality sorting' of MHC molecules.

It appears that the MHC class I HC behaves in a similar manner to other nascent glycoproteins, forming mixed disulphide intermediates with ERp57 in the context of a calnexin-calreticulin interaction. It is important to distinguish this general chaperone interaction from that interaction that is specific to MHC at the PLC. The role of calnexin in this early folding cascade suggests that redundancy of thiol oxidase activity is possible (i.e. ERp72 substituting for ERp57). It is probable that calreticulin has less of a role to play in the early folding of MHC class I HC and that calnexin is key for ERp57 recruitment and assisted function. These early events may now be looked at in a murine calreticulin knock out cell line. Finally it would also be of interest to assess the efficiency of β_2M association and HC oxidative state of in a glycosylation mutant of a human MHC class I HC. As discussed previously with the experiments on tunicamycin treated cells (Neefjes and Ploegh, 1988) glycoprotein independent pathways for disulphide rearrangements may be utilised for certain alleles.

THE FIRST HETERODIMERIC MHC CLASS I COMPLEX→ CHECKPOINT 1

Following the assembly of HC and β_2M, to form a correctly disulphide bonded heterodimer, a number of possibilities for further assembly are available. The key omission in the current experimental approach to MHC assembly is a reagent that can distinguish peptide occupied from peptide receptive MHC/ β_2M heterodimers. I have made some attempt to address this issue within my thesis with regard to the differing thermostability profiles of such complexes. Previously, β_2M associated complexes that have been unable to exit the ER have been considered as peptide empty complexes. This has been based upon original work in the TAP deficient cell lines where the egress of MHC class I molecules had been prevented (Baas et al., 1992; Ljunggren et al., 1990; Schumacher et al., 1990). This link between a failure of peptide supply/occupancy and egress initially supported the idea that these complexes were empty. Indeed, culturing such cell lines at 26^0C restored MHC class I expression and this was initially thought to represent the expression of empty molecules. Subsequent studies have isolated peptides from these 26^0C (37^0C unstable) complexes (De Silva et al., 1999), supporting the concept that MHC class I complexes may be loaded with a suboptimal/thermo-unstable/low affinity ligand cargo (Lewis and Elliott, 1998). There are some key conceptual difficulties with the notion of a suboptimally loaded complex that is not exported at physiological temperatures but that is exported at reduced temperatures. Is

export competence governed by a quality control folding sensor that is 'tricked' at 26^0C or is such machinery inoperative at this temperature? An alternative explanation that such complexes are better identified at reduced temperatures as they are less prone to dissociation. A principle that is applicable to most secretory glycoproteins is one where incompletely folded molecules are distinguished from compact molecules by UGGT and subsequently tagged by glucosylation. This 'glucosylation stamp' is a signal for further engagement within the calnexin/calreticulin cycle. However studies on the mouse allele D^b in wild type cells or peptide deficient cells with glycan radiolabelling shows less than 5% of all retained complexes are monoglucosylated (Hayes et al., 1995). The majority are Man $_{8\text{-}9}$GlcNAc$_2$ in the peptide deficient RMA/S cell line whilst in the wild type cell they have been acted upon the medial golgi enzyme alpha mannosidase II (resistant to endoglycosidase H). In a more recent investigation, the HC glycans within the PLC were analysed. This suggested that 50% of the HC/ β_2M complexes within the PLC were monoglucosylated (Radcliffe et al., 2002). It was suggested that the deglucosylated 50% is peptide occupied and awaiting release. Furthermore the complexes on the PLC show evidence of trimming to Man$_{6\text{-}8}$GlcNAc$_2$ forms, consistent with ongoing ER mannosidase activity. Therefore a UGGT folding sensor system may operate for complexes on the PLC. Indeed the steady state detection of monoglucosylated proteins is in equilibrium between glucosidase II mediated deglucosylation and UGGT mediated re-glucosylation. The ability of MHC class I complexes to bind to the PLC may alter this equilibrium so that the monoglucosylated from is more readily

identifiable. What about complexes that are retained in the peptide incompetent cell lines? In such cell lines MHC HC/ β_2M heterodimers are identified within the ER but their half life is faster than in peptide competent cells. This turnover is thought to be mediated through a reduced HC/ β_2M stability in the absence of peptide which leads to dissociation. Such misfolded HC, following reglucosylation, may then be a ligand for further calnexin binding. The process of glycoprotein loss through ERAD has been recently enlightened with the identification of EDEM (ER degradation enhancing alpha-mannosidase-like protein) (Molinari et al., 2003; Oda et al., 2003). The failure of a MHC class I glycoprotein to be re-glucosylated may target it for a degradative pathway which prevents its egress from the ER. This has recently been shown for non-MHC class I glycoproteins such as alpha 1 antitrypsin (Liu et al., 1999). The key change within a glycoprotein that is destined for degradation appears to be its conversion to a Man_8 form (Tsai et al., 2002). This is the signature for subsequent ERAD. This latter process appears to involve an ER resident alpha –mannosidase homologue that lacks enzyme activity. This protein has been termed EDEM (ER degradation enhancing alpha-mannosidase-like protein) and is a class II transmembrane protein with specificity for non-glucosylated misfolded Man_8 glycoproteins. Through the pathway of calnexin association, glucosidase II mediated calnexin release and ER mannosidase I modification, the misfolded glycoprotein is targeted for Sec 61 retrotranslocon transport and degradation, rather any than further UGGT mediated folding cycles. Therefore HC/ β_2M complexes (or free heavy chains) may have an initial quality checkpoint

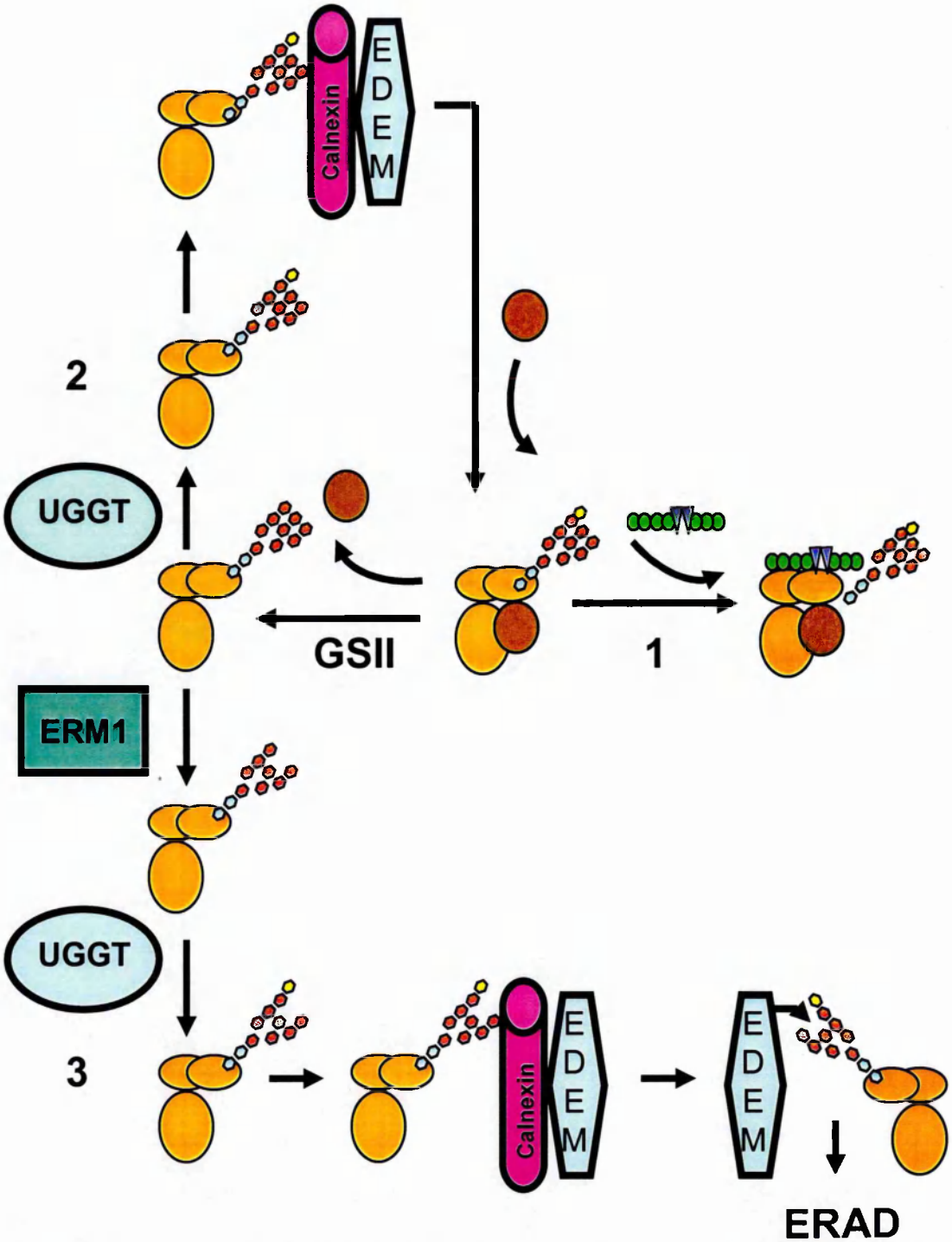


Figure 5.5.2 – The MHC:β2M complex - Checkpoint 1

The first quality checkpoint for MHC class I assembly may utilise the CNX/CRT/UGGT pathway with 3 options available to an empty MHC class I complex. It may bind peptide (1), dissociate to a free HC which re-enters the cycle (2) or be acted upon by ERM1 and enter a EDEM associated ERAD pathway (3).

1 that operates at the level of HC dissociation from calnexin. This is depicted in Figure 5.5.2 where there are 3 options for a HC/β_2M complex in a peptide incompetent cell. The first is the attainment of a degree of stability through an interaction with suboptimal peptide that maintains the HC/ β_2M association. This trimeric complex may not be a substrate for ER mannosidase I and hence escape ERAD. It is possible that it is a substrate for PLC binding upon which optimisation of the peptide cargo may occur. The second option is of rapid dissociation of HC from β_2M, re-glucosylation via UGGT and further calnexin binding. A further attempt at cooperative β_2M and peptide binding may then be made. The third option operates as a competition between EDEM binding or a further cycle of β_2M assembly/disassociation and UGGT mediated glyosylation. Binding to EDEM is facilitated by calnexin dependent ERM 1 activity to form a Man_8 HC. This mannose motif is recognised by EDEM and less favoured as a re-glycosylation motif by UGGT. This third option would ultimately lead to HC removal via ERAD.

Therefore checkpoint 1 is the measure of the cooperative stability of the HC / β_2M complex with the competing forces of

(i) Peptide mediated stabilisation of the MHC/ β_2M complex prior to further exchange/trimming or export

(ii) UGGT mediated reglucosylation of dissociated HC prior to a for a further calnexin cycle or

(iii) ER mannosidase I mediated Man_8 modification and calnexin/EDEM facilitated removal of the dissociated heavy chain.

THE FIRST HETEROTRIMERIC MHC CLASS I COMPLEX→ CHECKPOINT 2

Returning to the intermediate stages of MHC class I assembly following release from calnexin it is next convenient to consider 2 possibilities. The first possibility is that peptide acquisition occurs in the absence of further chaperone assistance and this completes the assembly process of the heterotrimeric complex (See 5.2). This type of complex is exemplified by MHC class I HC mutants with single amino acid changes that impair associations within the PLC. Also some naturally occurring alleles associate poorly with the PLC. For such alleles it is convenient to consider them as peptide receptive and perhaps open to chaperone independent sampling of peptides. B*2705 and B*4405 might behave in this manner, with the monoglucosylated trimeric complex subsequently acted upon by GS II. This compact molecule would no longer be a substrate for UGGT, passing quality control checkpoint 1 and able to leave the ER. The second possible intermediate stage of MHC class I assembly requires the peptide receptive complex to utilise the PLC to facilitate peptide loading. This assistance may then furnish the peptide receptive complex with a suitable peptide that permits egress from the ER. What happens to PLC dependent β_2M associated complexes (i.e. B*4402) in tapasin incompetent cell lines? For classical alleles such as B*4402 their inability to egress the ER leads to their eventual degradation through ERAD. As peptides are available in such tapasin incompetent cell lines and B*4402 molecules are able to load peptides in a

detergent lysate in the absence of tapasin, why is there a 'reluctance' to load in the ER? From the observations made within this thesis I have proposed one model that might explain this phenomenon. This model is built on 3 experimental observations and interpretations.

1) The first relates to the change in thermostability of B*4402 complexes in the presence and absence of ICP47.

2) The second is the improved thermostability of B*2705 over B*4402 in the absence of peptide and tapasin.

3) The third observation is from the MHC class II literature which relates the thermostability, SDS stability and compact conformation of different MHC class II molecules with their DM dependence and peptide receptivity.

One model which I have proposed from the observations made within this thesis is seen in Figure 5.5.3. The features of this model are: (i) MHC class I complexes may exist in an open conformation with or without peptide and a closed conformation with and without peptide (ii) different alleles may show different intrinsic propensities to adopt one of these conformations. We can consider an allele such as B*4402 as easily triggered to undergo a transition to a closed conformation following an interaction with suboptimal ligands that have a fast off rate and poor complementarity for anchor:pocket interactions. B*2705 may be more compact due to the composition of its binding pockets and be less easily triggered into a full rearrangement by poor affinity ligands. This more compact open conformation may be a peptide receptive form which upon suitable

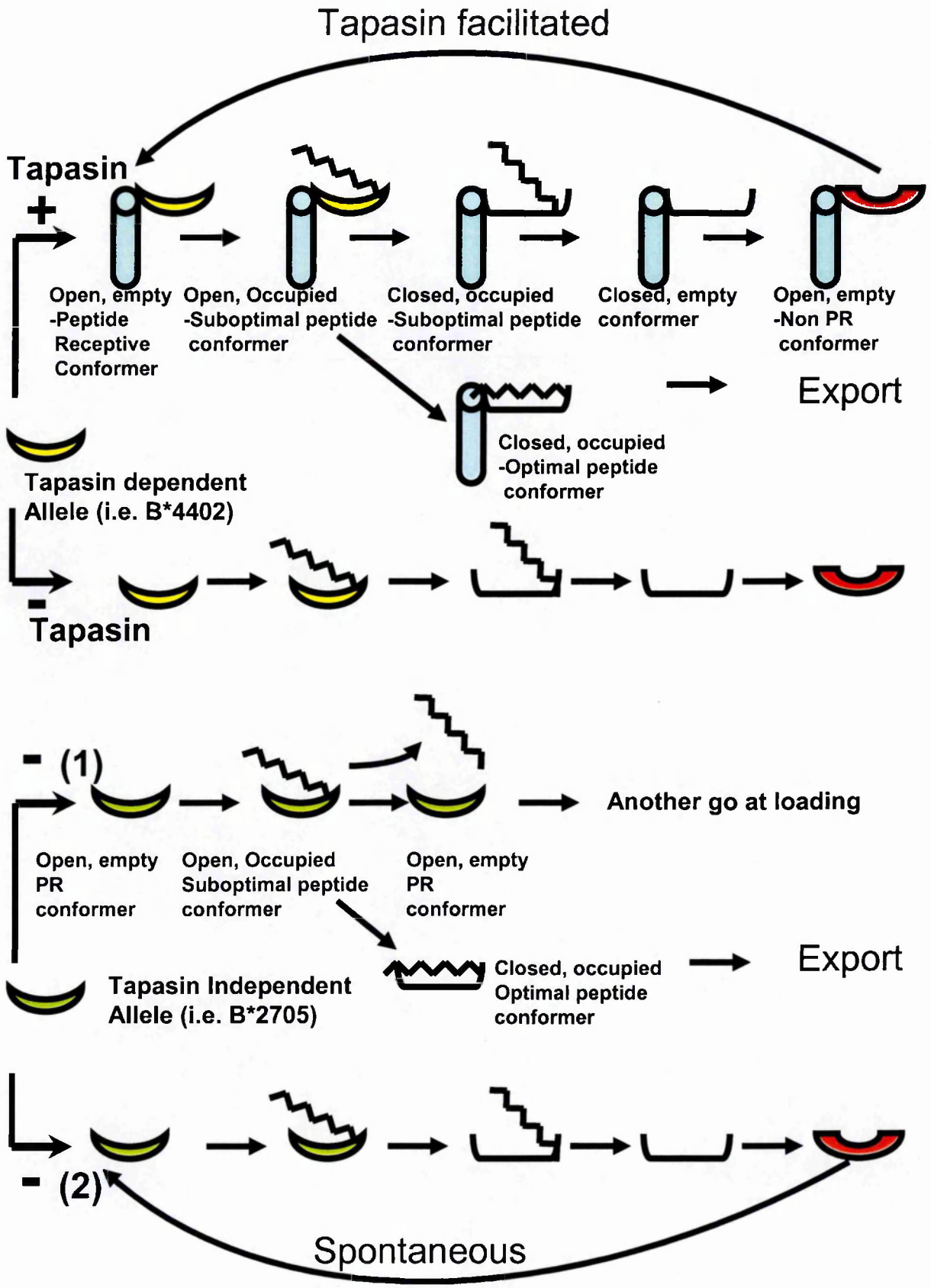


Figure 5.5.3 – Hypothetical model of the allele dependent activity of Tapasin in MHC class I assembly.

peptide occupation undergoes a full conformational change to a compact, closed peptide occupied form. In contrast, B*4402 may more easily move to a closed peptide vacant form which is unsuitable for further peptide binding. Such conformed but unreceptive molecules would eventually dissociate to HC and β_2M and be dealt with through ERAD. The role of tapasin may be to disfavour the non-productive conformational change from a sub optimal peptide occupied open conformer to a peptide dissociated closed conformation. This may be secondary to an affinity for the open conformation which alters the sensitivity for a full conformational change by a ligand that is suboptimal. This would predict that tapasin dependent alleles would acquire peptides of a higher affinity than alleles that can acquire peptides equally well in the absence or presence of tapasin. In the latter case such alleles would have a mix of affinities, with the introduction of tapasin competing with peptide for a productive interaction with open conformation complexes. This is consistent with the results seen in thesis with regard to B*4402 and B*2705. An additional function of tapasin for such dependent alleles may be to act upon open, peptide empty non receptive conformers to return them to a peptide receptive state. Whereas B*4402 complexes are unable to load peptide in the absence of tapasin inside a cell, such complexes can subsequently be loaded with peptide in a detergent lysate. This is analogous to the detergent C_8Glc in MHC class II assembly. Here the detergent is able to liberate CLIP peptide ('suboptimal ligand') which releases peptide receptive MHC Class II molecules which were previously difficult to load in the absence of HLA-DM.

For nonclassical alleles such as HLA-G a different process appears to take place. Elegant studies by Ahn and colleagues have shown that HLA-G is poorly expressed at the cell surface in tapasin incompetent cell lines and that such complexes are probably occupied with suboptimal complexes (Park and Ahn, 2003). This latter point is based upon the observations that ICP47 blockade further reduces the thermostability of HLA-G molecules and that high affinity peptides improved export in the presence of tapasin. The unique feature of HLA-G is the presence of an RKKSSD motif in its shortened cytoplasmic tail. This motif permits association with the coatmer complex COP1. COP1 recognition of the cytoplasmic tail of HLA-G directs its retrieval of the heterotrimeric complex from the ERGIC to the ER. These complexes have not yet attained Endo H resistance and are therefore proximal to the medial golgi. It is hypothesised that this pathway is dependent upon suboptimal ligand occupation of the MHC class I complex since HC/ β_2M complexes are more rapidly lost in peptide incompetent cells possibly relating to their inability to enter this ER exit and retrieval pathway, which in some way protects them from ERAD (even though they are not suitable for medial golgi egress). Similar studies were undertaken over 10 years ago in a peptide incompetent cell where it was first suggested that MHC class I complexes may recycle between the ER and cis-golgi (Hsu et al., 1991). These studies were undertaken in a mouse cell line and showed co-localisation of a classical mouse MHC class I allele with a marker of recycling vesicles. Such studies have not been undertaken for human alleles, at least not in the context of

a peptide incompetent cell, and may therefore add further to the recycling mechanisms that operate for classical molecules under different circumstances of peptide availability or 'loadability'. Another non ciassical mouse allele, H2-M3, which binds selected N-formylated peptides has been similarly assessed (Chun et al., 2001). Again, the provision of high affinity ligands is necessary but not sufficient for cell surface expression requiring tapasin for loading. It is proposed that suboptimal ligands are required for protection of HC from degradation. It will be interesting to see how this is mediated as H2-M3 does not possess an ER retrieval motif in its cytoplasmic tail and is therefore is unlikely to recycle in an identical manner to HLA –G. It is possible that it utilises the retrieval motif of another chaperone such as calreticulin (i.e. driven by the KDELR) (Yamamoto et al., 2001) or undergoes COPI independent retrieval (Girod et al., 1999; Storrie et al., 2000). Alternatively suboptimal peptide may operate differentiy for H2-M3 and simply extend the HC/β_2M interaction thus preventing premature HC dissociation and ERAD mediated degradation.

In considering these experiments upon classical and non-classical MHC class I complexes a further quality control checkpoint is suggested. Such a check point permits HLA-G complexes with high affinity peptide to egress and peptide occupied H2-M3 and classical alleles in TAP incompetent cells to be identified and retained. A further consideration with regard to this hypothetical checkpoint is the behaviour of the MHC class I mutant T134K A*0201 (A2). This allele exits with a suboptimal peptide cargo, cannot interact with the PLC and traffics faster

than wild type A2 (Lewis et al., 1996; Peace-Brewer et al., 1996). However, in the presence of a TAP blockade it is retained in the ER suggesting that suboptimal ligands are important for bypassing checkpoint 1 and in their absence the class I complex is unstable and dissociated HC may interact with the calnexin EDEM apparatus for retention and removal (Lewis and Elliott, 1998). Similarly, its trafficking and poorer peptide selection compared to wild type in the presence of suboptimal ligand suggests that it may selectively bypass checkpoint 2. This hypothetical checkpoint is presumably active upon the HLA-G and H2-M3 complexes mentioned earlier. Would T134K mutations in HLA-G permit suboptimal complex egress increase the trafficking rate or conversely slow the trafficking rate of HLA-G secondary to an antagonism of tapasin facilitated high affinity peptide loading ? Such a checkpoint 2 may also operate upon classical MHC class I complexes (Paulsson et al., 2002). It is intriguing that wild type A2 expressed in tapasin incompetent cell lines behaves differently to the non tapasin binding T134K allele (Lewis et al., 1998). Here the trafficking time is not reduced and the complexes that do exit are more stable. This would suggest that the ability to associate with tapasin is indirectly involved with checkpoint 2, secondary to its effect upon peptide loading, but that the quality control checkpoint of the loaded complex is not tapasin itself. The molecular nature of this secondary checkpoint is not clear although experiments with HLA-G offer some interesting perspectives. When the cytoplasmic tail of G is swapped for that of A2 the retention of G is no longer seen. As the peptide repertoire is not expected to change (assumption at present), it is probable that the previous

cargo which was being rejected at checkpoint 2 is now able to egress the ER when the competing ER retention is removed. Similarly, the reverse is seen with a G tail upon A2. Therefore peptide loading may be sufficient for default egress unless a specific signal for retrieval is received. Alternatively peptide loading may engage an ER export apparatus whose activity is impaired by chaperone/COP1 association. Recent studies upon the ER export of potassium channels have identified an important role for phosphorylation of a cytoplasmic tail sequence. This sequence, when phosphorylated, is able to bind a member of the 14-3-3 family of proteins which permits ER release (O'Kelly et al., 2002). This 14-3-3 binding is shown to block binding COP1 to the cytoplasmic tail of the potassium channel. Such a conserved phosphorylation sequence is seen for MHC class I heavy chains, including HLA-G (Vega and Strominger, 1989). In the case of T134K, a gain of function may be introduced with a pro-exit phenotype acquired in the absence of 'correct' assembly. Another possibility with regard to the latter allele is that a different ER exit route is chosen from wild type MHC class I complexes. Edidin and colleagues have provided some experimental data to support this latter hypothesis through the use of fluorescently tagged complexes. Through the analysis of FRET between complexes they suggest that T134K complexes do not co-localise with wild type complexes and may leave the ER through a different exit site (Pentcheva and Edidin, 2001).

In the case of alleles such as B*4402, peptide loading at the PLC is mandatory for competent assembly. For alleles such as B*2705, such an interaction is

possible and indeed is shown within this thesis to increase the rate and extent of peptide loading despite its previous tapasin independent status. This redundancy of chaperone requirement may be important for the immune response to pathogens that subvert PLC interactions. Such infections would in principle, impair the ability of certain alleles to present pathogen peptides to CTL. In contrast, certain alleles may require chaperone assistance to load pathogenic peptides with the slowest off rate, thus providing a longer cell surface appearance and opportunity for recognition and CTL generation. For alleles with a restricted peptide display that is distinct for particular receptors (i.e. HLA-E and CD94/NKG2A) it may be beneficial to become tapasin dependent so that only a particular set of peptides mediates cell surface expression. The molecular mechanisms that mediate such peptide loading at the PLC are far from clear. Within my thesis I have shown that the thermostability of a complex is greatest when loaded with the assistance of tapasin at the PLC interface. This effect may relate to the abundance of peptides at the PLC, or perhaps favoured peptide trimming at the PLC. Interference with other components of the PLC has also been achieved. When calreticulin is absent from the loading complex a greater proportion of sub-optimally loaded complexes leave the ER (Gao et al., 2002). However, the PLC shows association of tapasin, heavy chain and ERp57 within this modified PLC. How the absence of calreticulin leads to impaired optimisation is not understood but may relate to:

(1) a stabilisation effect upon the MHC class I/TAP/tapasin complex,

(2) modulation of a calcium dependent ER trimmase,

(3) chaperone of peptides from TAP,

(4) retrieval of suboptimally loaded complexes via its KDEL motif or

(5) augmentation of the action of tapasin.

It will be interesting to analyse human tapasin dependent and independent alleles in the absence of calreticulin and assess whether a correlation exists between tapasin and calreticulin dependence. The third important component of the loading complex is ERp57. Insightful experiments undertaken by Cresswell and colleagues have drawn attention to this role of disulphide isomerisation in peptide loading at the PLC. Their first key observation was that of a mixed disulphide complex of tapasin and ERp57 within the PLC (Dick et al., 2002). The entire PLC associated ERp57 was disulphide linked to tapasin, whereas approximately 50 % of PLC associated tapasin was disulphide linked to ERp57. When ERp57 was excluded from the complex through the clever generation of a tapasin mutant (C95A), B*4402 complexes showed a slightly reduced thermostability compared to wild type tapasin. In addition, the HC within the PLC was partially reduced in the presence of the C95A mutant of tapasin. If it is assumed that the MHC class I HC/ β_2M complex is fully oxidised prior to the PLC interaction, it is then implied that reduction of one the disulphide bridges in the MHC class I HC takes place within the PLC. The disulphide bridge in the α2 domain would seem the obvious candidate for an influence upon peptide loading through isomerisation. MHC class I mutants which are mutated at either the Cys 101 or 164 sites within the α2 domain are poorly peptide receptive (Warburton et al., 1994) as might be expected as the disulphide bridge is important in the final

structure. How this process of heavy chain disulphide bond isomerisation contributes to MHC class I peptide assembly and optimisation is not clear. Experiments by Powis and colleagues have shown that peptide unoccupied complexes are move susceptible to recombinant ERp57 mediated reduction (Antoniou et al., 2002). Perhaps the susceptibility of cys 101-164 reduction is related to the conformation of the peptide biding groove and accessibility to the reducing agent. This may also differ between alleles so that certain alleles are more dependent upon a tapasin-ERp57 mediated oxidation event. Such oxidation would allow for further peptide sampling and its absence might 'fix' the dependent allele into a partially reduced non peptide receptive confirmation.

The complexity of chaperone interactions within the PLC and their cooperative and unique roles in the assembly of certain MHC class I molecules cannot be overstated. The involvement of pathways relating to glycoprotein folding, oxido-reductase activity and perhaps the unique roles of calreticulin and tapasin still remain poorly integrated in providing an explanation as to how an optimised peptide repertoire following PLC association.

5.6 CONCLUSIONS

At the outset of my research there were 13 published papers concerning the role of tapasin in antigen processing (now 158 in 4 years). From this initial experimental database the functions of tapasin as a peptide editor/peptide trimmase/MHC foldase and 'PLC cornerstone' were proposed. My experimental

objectives were to assess the allele dependencies upon tapasin, initially through a genetic approach and then through a functional one. The first of these approaches confirmed that tapasin in not polymorphic, perhaps suggesting that allelic dependencies/independencies are reflective of MHC variation conserved. The functional approach has been constructed around the tapasin dependent (B*4402) and independent alleles (B*2705). By developing a time resolved assay that is reflective of average peptide repertoire I have been able to further dissect the similarities and differences of these alleles within different environments. The resulting experimental observations have suggested that tapasin dependent non-trafficking alleles may not be peptide empty and that tapasin independent alleles are kinetically and qualitatively disadvantaged when they acquire peptide in the absence of tapasin assistance. Aiso a phenomena of continued thermostability improvement has been noted which occurs at a time when the complexes have left the ER environment. Lastly, I have demonstrated that maximal optimisation of the MHC class I cargo is achieved when the MHC class I complex abridges to the PLC and that a singie amino acid change in the F pocket of a tapasin dependent allele can reverse the previously dependent phenotype. These observations have led me to reconsider some of the pre-existing concepts regarding MHC class I peptide acquisition, intracellular trafficking and chaperone functions. These evolving ideas will now form the basis of further work to establish, at a moiecular level, how tapasin influences peptide binding by MHC class I molecules and which MHC class I structural features contribute to this dependence and independence. Finally, the

mechanisms governing quality control at checkpoint 2 and post ER optimisation are undetermined at present. The details of such mechanisms are warranted both for an improved understanding of both classical MHC class I presentation and possibly, cross presentation. Extending these investigations to dendritic cells will be a considerable challenge over the next few years. Such an understanding of MHC I antigen presentation pathways within dendritic cells will be of crucial importance, as we attempt to influence the priming and generation of effector/memory T cell responses against a wide variety of human diseases (viral and bacterial infections, solid tumours and viral associated tumours).

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